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14. ABSTRACT

Increased expression of eIF4E occurs in breast cancers and contributes to carcinogenesis by stimulating expression of cancer-related genes. However, eIF4E activity is a function of activating and inhibitory influences determined by expression and phosphorylation of eIF4E and eIF4E-binding proteins (4E-BPs). Rapamycin and everolimus are potential cancer drugs that target eIF4E indirectly by modifying 4E-BP function. Our hypothesis was that combined analyses of eIF4E and 4E-BPs would allow insights into eIF4E activity and into which tumours will respond to eIF4E-directed therapies.

We determined expression of eIF4E, 4E-BP1 and 2, and phosph-4E-BP1 in breast tumours using immunohistochemistry (IHC). Data were analysed to estimate eIF4E activity. eIF4E activity estimates gave greater prognostic insights than analysis of eIF4E alone. In cell lines we used a reporter to measure eIF4E activity and showed that this was a significant predictor of rapamycin sensitivity. However, IHC based estimates of eIF4E activity in breast cancer biopsies taken at diagnosis did not predict sensitivity to pre-operative treatment with rapamycin derivative everolimus. An explanation for this lack of correlation was that tumours with higher pre-treatment eIF4E activity undergo potent and rapid selection for everolimus resistance.

We concluded that estimates of eIF4E activity have prognostic value in breast cancer but little predictive value for everolimus therapy. Acquired resistance may limit the worth of everolimus monotherapy in breast cancer treatment.

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Appendices:

i) **Coleman et al (2009) Br J Cancer 100, 1393-9**
ii) **Smith et al (2010) Biochem J 429, 283-290**
iii) **Satheesha et al (2011) Mol Cancer 10, 19**

1. Introduction

Increased expression of eIF4E has frequently been reported in breast cancers and is thought to make fundamental contributions to disease development and progression[1]. Increased eIF4E activity acts to enhance the translation of cancer-related transcripts that contain highly structured 5' untranslated regions (UTRs) in their mRNAs. Over-expression of eIF4E has been shown to correlate with poor prognosis in breast cancer[2] therefore the level of eIF4E over-expression has been studied extensively as a prognostic marker with some success. Furthermore, eIF4E is an established target for cancer therapy[3] and clinical trials of the efficacy and safety of cancer therapeutics that target eIF4E have been carried out, again, with some success. However, it is clear that eIF4E expression does not equate to eIF4E activity, since eIF4E activity is additionally regulated by a family of binding proteins, the 4E-BPs, that bind to and inhibit eIF4E activity[4]. Our hypothesis was that analysis of eIF4E *activity* in individual breast tumours, as opposed to eIF4E *expression*, gives improved prognostic, predictive and biological understanding of individual breast cancers, and overall insights into the mechanisms of breast carcinogenesis.

In order to test this hypothesis we aimed to investigate eIF4E activity in breast carcinogenesis using a novel cross-disciplinary approach. First, we aimed to determine expression levels of eIF4E and its regulators in breast tumours, and to estimate the individual contributions of each factor to eIF4E activity by relating each expression level to cancer survival. This approach would allow us to build, in collaboration with mathematicians, an equation relating the levels of these factors together to estimate eIF4E activity. Next we aimed to validate these mathematical relationships in tissue culture systems where we would be able to measure both expression levels and eIF4E activity. Finally, we aimed to determine whether measures of eIF4E activity in cell lines, and estimates of eIF4E activity in tumours, would act as predictive markers for cellular responses to eIF4E directed therapies.

2. Body

2.1 Examination of archival breast tumours (Statement of Work A1, A2, B1, B4).

We have determined expression levels of eIF4E, 4E-BP1, 4E-BP2 and phospho-4E-BP1 Thr37/46 (p-4E-BP1) by immuno-histochemistry (IHC) in breast tumours from a cohort of 424 patients, supported by extensive clinical background and follow up. We have been unable to demonstrate significant expression of 4E-BP3 mRNA (qPCR) or 4E-BP3 protein (appropriately specific immuno-reactivity in IHC) in breast tissue. Consequently 4E-BP3 has not been included in our analyses. We have established the relationship between expression of these markers and tumour grade, size and type. Moreover, we have combined the insights gained from each of these markers into an estimate of eIF4E activity. We have developed a mathematical function that relates expression levels of eIF4E, 4E-BP1, 4E-BP2 and p-4E-BP1 in breast tumours to survival. This function, '*z*', provides additional prognostic insights when compared to examination of eIF4E expression levels alone. This variable can be described as X–B1/4+PB1/2-B2/4, where X, B1, PB1 and B2 represent eIF4E, 4E-BP1, p-4E-BP1 and 4E-BP2 levels respectively. This work has been published[5] (manuscript attached within appendices).

2.2 Determination of expression levels of eIF4E and its regulators, and estimation of eIF4E activity, in cell lines (SoW A3, B2).

Next, we were interested to determine the validity of our estimate of eIF4E activity. We aimed to study tissue culture cells in which we would be able to determine both expression levels of eIF4E and its regulators (this section), and actual eIF4E activity (section 2.3).

We determined relative expression levels of eIF4E, 4E-BP1, 4E-BP2 and p-4E-BP1 in a panel of human cell lines (SW480, Caco-2, U2020, H1299, A549, HB2, MDA-MB-231, MCF-10A, MCF7) using Western blotting and densitometry. Two alternative cell lysis and normalisation methods were used: RIPA lysis normalized to total protein (Figure 1), and Laemmli lysis normalized to expression of beta-actin (data not shown). In order to reproduce the quantification that was used for breast tumours (section 2.1), expression of the markers in the cells lines was also determined using IHC. We found no correlation between assessments of relative expression by Western and IHC (Figure 1). It is worth noting that assessment by IHC in cell lines proved particularly problematic. We found

marked heterogeneity of expression throughout the cell populations and cellular morphology was poorly maintained making scoring especially difficult.

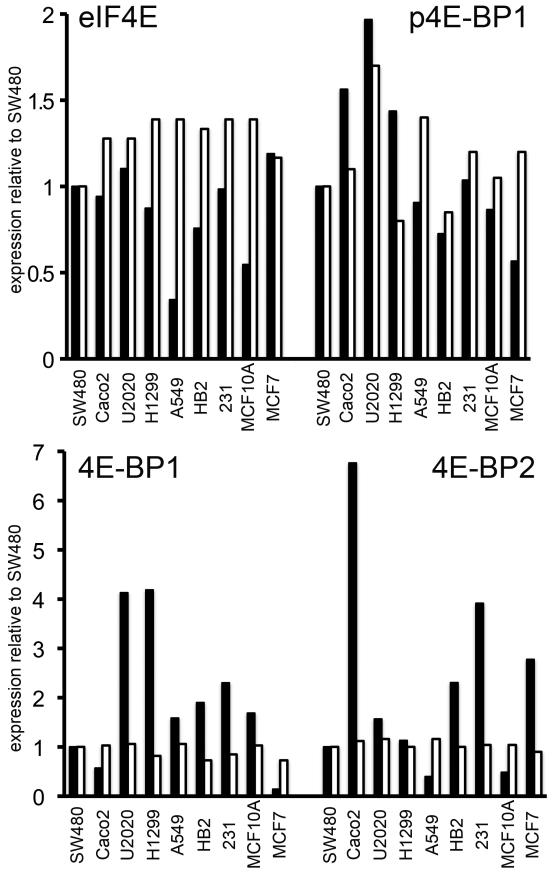

Figure 1 Expression levels of eIF4E and its regulators as determined by Western blot and densitometry (filled bars) or IHC (open bars) do not correlate. Expression levels of eIF4E, 4E-BP1, p-4E-BP1 and 4E-BP2 in a panel of human cell lines were determined by densitometric analyses of Western blots of RIPA extracts normalized to total protein, or by semi-quantitative scoring of intensity and the proportion of cells showing positivity after immuno-histochemical staining of agarose encased and formalin fixed paraffin embedded cells. Data were normalised to values from SW480 cells to allow comparisons.

Next, we determined estimates of eIF4E activity in these cell lines by calculating *z* (see section 2.1) using the expression data from Western blots or IHC. This is not a trivial procedure for the data from Western blots. Densitometry values from Western blots were first mapped to values that were within an appropriate range for use with the *z* function – ie from 0 to 7. However, simply linearly transforming densitometry values to fit within this range defining the lowest as 0 and the maximum value as 7 does not represent a likely solution with only nine cell lines, since scores of 0 and 7 occur rarely in tumours. We transformed the maximum value in the cell lines to the most common positive value in the

tumours (eIF4E: 5; 4E-BP1: 6; p4E-BP1: 5; 4E-BP2: 5) and have maintained the fold differences to this for the other cell lines. As expected, considering the differences in the assessments of expression levels by Western and IHC, there was no significant correlation between the *z* values determined from these two datasets (Figure 2).

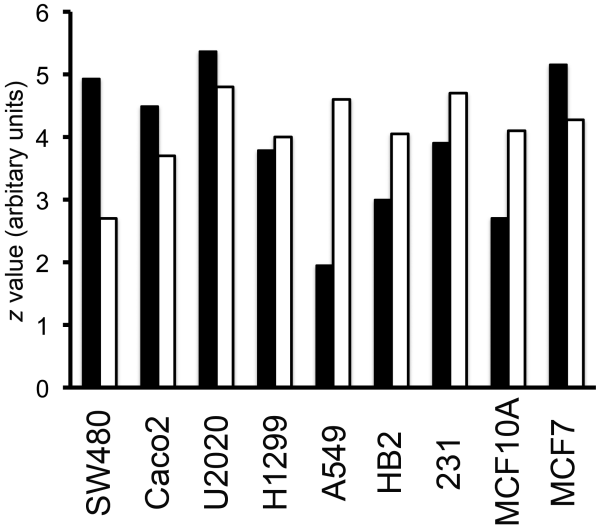

Figure 2 Estimated eIF4E activities based on data from Western blots (filled bars) or IHC (open bars) do not correlate. eIF4E activities were estimated from expression data for eIF4E, 4E-BP1, p-4E-BP1 and 4E-BP2 using the function z^5.

2.3 Determination of eIF4E activity in cell lines (SoW A4, A5, B3).

The cellular role of eIF4E is to bind to mRNA caps allowing recruitment of eIF4F, and subsequently the translational machinery. The complex formed scans linearly along the 5'UTR until an initiation codon in good context is encountered, at which point further elements of translational machinery are recruited and protein synthesis starts. Some 5'UTRs, frequently those on cancer-related transcripts, contain a high degree of secondary structure that can inhibit cap-recognition and translational scanning. Increased eIF4E activity is thought to reduce the effects of 5'UTR structure by enhancing cap-recognition and scanning, therefore increasing translation of these specific oncogenic transcripts[1]. Having produced two different estimates of eIF4E activities in cell lines (Figure 2), we next wished to establish the accuracy of the estimates. Therefore, we aimed to measure actual eIF4E activity experimentally. Two techniques to measure eIF4E activity were used.

Firstly, we used a fluorescent reporter assay. We have previously shown that a 5'UTR expressed from the human *AXIN2* gene contains a sixty nucleotide sequence that is predicted to form a stable stem-loop structure[6]. This structure fits the criteria associated with UTRs that determine differential translational efficiencies in response to changes in eIF4E activity[7], while lacking other motifs known to allow regulation of translation (e.g. upstream AUG codons or binding sites for trans-acting proteins). We now wished to examine whether the translational efficiency defined by this UTR would respond to experimentally induced changes in eIF4E activity, and could therefore be used to measure endogenous cellular eIF4E activity. The sequence was cloned upstream of the GFP reading frame in an expression vector. MCF7 cells were transiently transfected with an equal copy number of vectors to allow expression of GFP mRNAs with either a control non-regulatory 5'UTR or this structured 5'UTR, along with either empty expression plasmids or plasmids allowing eIF4E over-expression. GFP protein expression was measured by flow-cytometry and GFP mRNA expression was measured by qPCR allowing determination of relative translational efficiencies for each GFP message (Figure 3A). Western blot analyses were used to confirm expression of exogenous eIF4E in the appropriate co-transfected cells (Figure 3B). The translational efficiency of the control reporter was not significantly altered by eIF4E over-expression (compare lanes 1 and 2), demonstrating that eIF4E over-expression did not cause a general enhancement of translation. As previously reported[6], the structured 5'UTR conferred repression of translation (compare lanes 1 and 3; $p=0.002$). Critically, this repression was overcome by exogenous eIF4E (compare lanes 3 and 4; $p=0.002$), resulting in translation with the same efficiency as messages lacking inhibitory 5'UTRs. We concluded that this reporter did indeed respond to changes in eIF4E activity. Relative translational efficiencies specified by this eIF4E-responsive 5'UTR were determined in the panel of cell lines. Cells were transiently transfected with vectors to allow expression of GFP mRNAs with control or the structured 5'UTR as before, and translational efficiencies were determined (Figure 4A). A range of translational efficiencies was seen, with A549 cells determining the lowest, and HB2 cells the highest (Figure 4B).

Note that we have now employed the translational efficiency assay developed here for further published studies of UTR function[8] (manuscript attached within appendices).

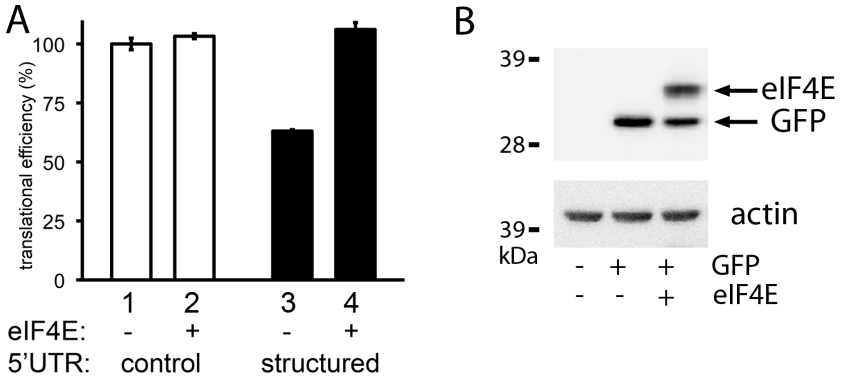

Figure 3 Translational efficiency specified by a structured 5'UTR reporter responds to changes in eIF4E activity. A) Reporters were constructed to express mRNAs containing the GFP reading frame preceded by a control 5'UTR lacking regulatory motifs (control) or a sequence predicted to form a stable stem-loop structure (structured). MCF7 cells were transiently transfected with equal copy numbers of either control or structured reporters along with either empty expression vector (-) or vector to allow over-expression of eIF4E (+). GFP protein and mRNA were quantified by flow-cytometry and real-time PCR respectively. Translational efficiency (protein synthesised per unit mRNA) is presented relative to the control. Data points represent means (+/-standard deviations) of technical triplicates within a representative experiment. B) Expression of exogenous proteins was confirmed by Western blot analysis using anti-HA (exogenous GFP and eIF4E both include the HA epitope).

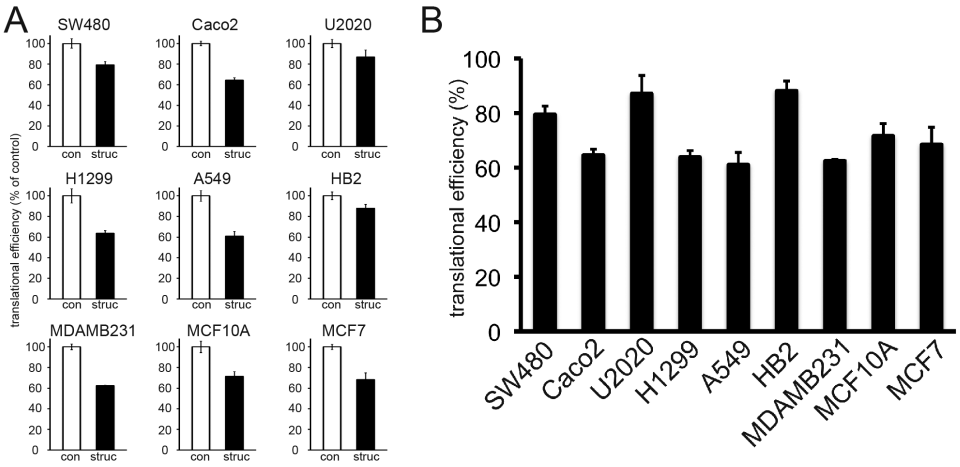

Figure 4 Translational efficiencies specified by a structured 5'UTR reporter vary in different cell lines. A) Cells were transiently transfected with equal copy numbers of plasmids to allow expression of transcripts with the GFP reading frame preceded by either a control 5'UTR lacking regulatory motifs (con) or a sequence predicted to form a stable stem-loop structure (struc). GFP protein and mRNA were quantified by flow-cytometry and real-time PCR respectively. Translational efficiency (protein synthesised per unit mRNA) is presented relative to the control. Data points represent means (+/-standard deviations) of technical triplicates within a representative experiment. A minimum of two independent experiments were performed. B) Translational efficiencies of transcripts with structured 5'UTRs in the 9 cells lines.

Secondly, we used an assay that allows quantification of eIF4E that is capable of binding to the mRNA cap. Methyl-7-GTP-sepharose was used to purify cap-binding eIF4E from lysates of each cell line containing equal masses of total protein; eIF4E recovered was quantified using Western blotting and densitometry (Figure 5A). These values represent relative quantification of eIF4E that can bind mRNA caps within these cell lines, which could be thought of as 'eIF4E activity'. However, we found that different amounts of 4E-BP1 co-purified with eIF4E in each cell line (Figure 5B). Consequently, we concluded that some of the eIF4E that was active in terms of cap-binding would not have been able to assemble eIF4F and initiate translation. Furthermore, the proportions of this cap-binding but translationally-inactive eIF4E relative to total cap-binding eIF4E differed between cell lines. Therefore, we have not used this assay as a physiologically relevant measure of eIF4E activity in terms of the oncogenic function of enhancing translation of specific transcripts.

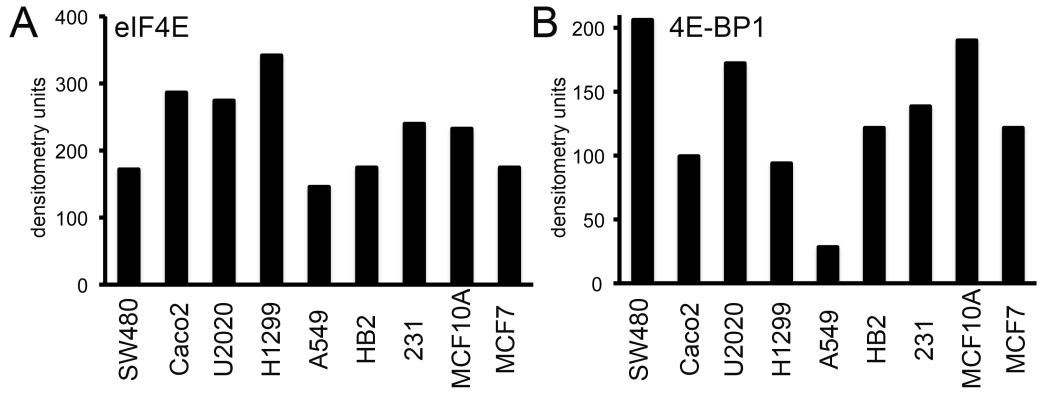

Figure 5 Cap-binding assays determine eIF4E's cap-recognition activity but, since 4E-BP1 is co-purified, this may not relate to eIF4E's ability to assemble active translation complexes. Proteins were purified on methyl-7-GTP-sepharose from cell lysates containing equal masses of total protein. Purified eIF4E (A) and 4E-BP1 (B) were quantified by Western blot and densitometry.

Estimates of eIF4E activity that were based on expression levels of eIF4E and its regulators (section 2.2), and the measures of eIF4E activity from the translational reporter assay (this section) were compared (Figure 6). No significant correlations were observed between estimates (black and open bars) and the actual measurement of eIF4E activity (grey bars) (Spearman's rank correlations: Western derived *z* with measured activity r=0.367, p=0.33;

IHC derived *z* with measured activity r=-0.161, p=0.678). The lack of correlation with the estimate derived from IHC expression data may reflect the inaccuracy of IHC assessment in the context of cell lines. The estimate derived from Western blot data, in fact, appears to be surprisingly accurate in five of the nine cell lines; this may suggest that further regulatory factors not taken into account in the current Western analyses may have critical roles in the other four cell lines.

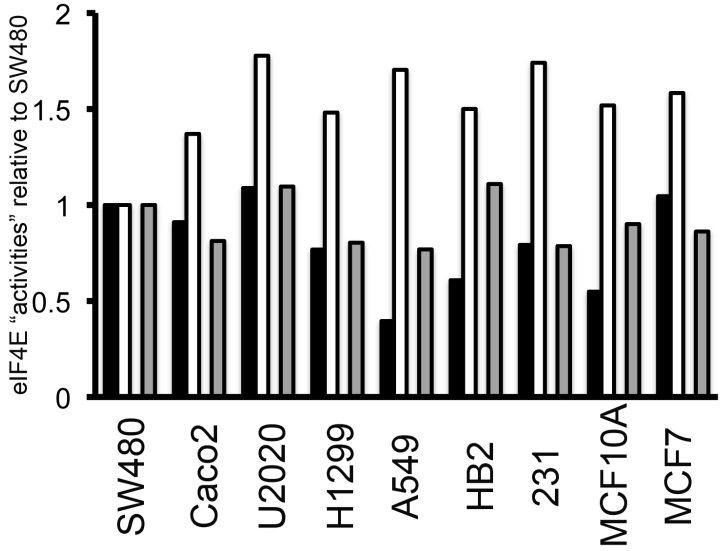

Figure 6 Estimated and experimentally determined eIF4E activities do not correlate. eIF4E activities were estimated using the *z* function (see Figure 2) on data derived from Western blots (black bars) or IHC (open bars). eIF4E activity was experimentally determined by analyses of the translational efficiency specified by a structured 5'UTR (grey bars; see Figure 4B). Data were normalised to values from SW480 cells to allow comparisons.

2.4 Analyses of the therapy predictive value of estimates of eIF4E activity in cell lines (SoW A1, B4).

Rapamycin, and derivatives such as everolimus (RAD001), have been tested as cancer therapeutics with some success[9]. The drugs inhibit the activity of the protein kinase mammalian Target Of Rapamycin (mTOR). It is well established that mTOR activity is up-regulated in many cancers[10]. Critical mTOR targets are 4E-BP1 and 2, which - when hyperphosphorylated by mTOR - are unable to interact with and inhibit eIF4E. Thus cells in which mTOR activity is high are thought to have high eIF4E activity. Our hypothesis was that high eIF4E activity would be associated with high sensitivity to mTOR inhibitors, as the cells would require continued mTOR activity to maintain their eIF4E-dependent aberrant growth and survival.

To test this hypothesis in cell lines, we treated our panel of cell lines with rapamycin and determined their relative sensitivities. Cells were treated with different doses of rapamycin and proliferation/survival was determined relative to control treated cells using MTT assays (Figure 7A). Sensitivities to the highest dose are shown in Figure 7B. A range of sensitivities was seen, with a three-fold difference between the most sensitive (MCF7) and most resistant (MDA-MB-231).

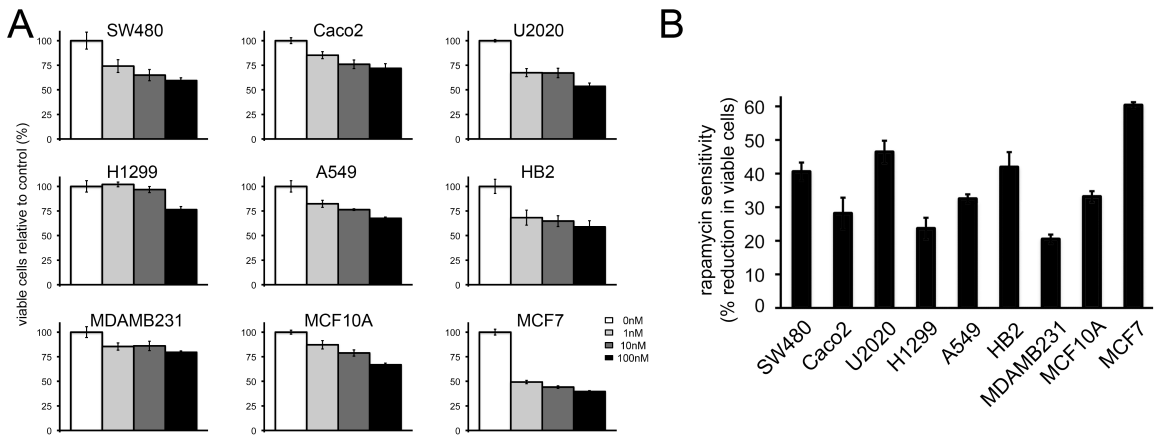

Figure 7 Cell lines have different sensitivities to rapamycin. A) Cells were treated with either control, or different doses of rapamycin and growth/proliferation was monitored using MTT assays. MTT readings after 48 hours are shown relative to control. B) Relative sensitivities to rapamycin are shown; these are the % reductions in growth/proliferation caused by 100nM rapamycin. Data points represent means (+/- standard deviations) from five technical replicates within a representative experiment. A minimum of two independent experiments were performed.

Sensitivities were compared with the eIF4E activities determined using the reporter assay (section 2.3). Initially, we analysed this relationship using Spearman's rank correlation coefficient; we found a strong and significant positive association ($r=0.72$; $p=0.037$). However, the correlation was particularly evident in 8 of the 9 cell lines; if MCF7 cells, which were more sensitive to rapamycin than predicted, were excluded from the analysis the strength and significance of the relationship was increased ($r=0.83$; $p=0.015$). Similarly in linear regression, a highly significant relationship was seen when MCF7 cells were excluded from the analysis (Figure 8; $p=0.0037$). We concluded that eIF4E activities as determined by our reporter assay predicted sensitivity to rapamycin in 8 of the 9 cell lines.

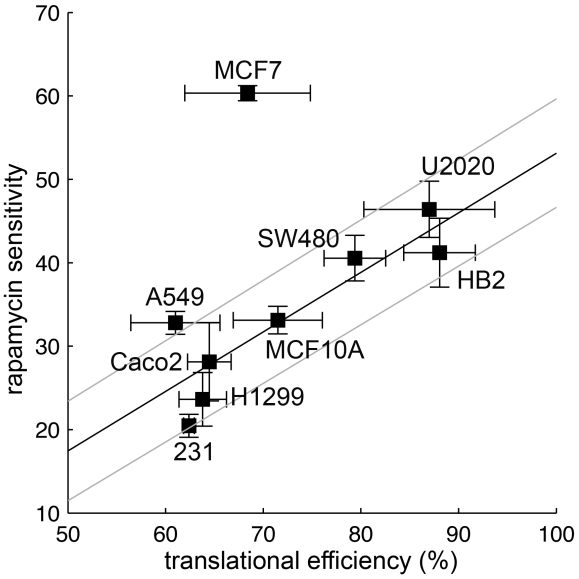

Figure 8 Experimentally determined eIF4E activities correlate with sensitivities to rapamycin. Data from Figure 4B (x-axis) and Figure 7B (y-axis) were plotted for each cell line. Linear regression was performed to determine the relationship in the cell lines excluding the outlier, MCF7 cells; the linear model is shown as a line (black) with 95% confidence intervals (grey lines) (p=0.0037).

2.5 Analyses of the therapy predictive value of estimates of eIF4E activity in tumours (SoW A1, B4).

Next, we examined whether our estimate of eIF4E activity, the *z* function, provided predictive insights with respect to response to eIF4E-targetted therapy in clinical breast cancers. Tumour samples from 23 breast cancer patients treated with at least 10 days neo-adjuvant everolimus were obtained both pre-treatment (core biopsies taken for diagnosis) and post-treatment (surgical resection samples) from collaborators (Prof John Bartlett, then in Edinburgh, UK but now at the Ontario Institute for Cancer Research, Canada). Expression of eIF4E, 4E-BP1, 4E-BP2 and p-4E-BP1 was quantified by IHC as before. Expression scores were used to estimate eIF4E activity using '*z*'. In addition, tumour cell proliferation was estimated in terms of Ki67 staining.

17/22 tumours showed reduced Ki67 scores after treatment (mean reduction 48%) indicating apparent responses to everolimus. Disappointingly, estimates of pre-treatment eIF4E activity did not predict the occurrence or extent of these responses. Similarly, pre-treatment phospho-4E-BP1 levels had no predictive value. Estimated eIF4E activity was,

however, reduced in post-treatment samples (mean change in score -1.7; range -8 to +1.25; $p<0.001$) but surprisingly this was not attributable to reduced phospho-4E-BP1. Phospho-4E-BP1 expression was reduced after treatment (mean -2.3; range -6 to +1; $p<0.001$), suggesting a reduction in mTOR-dependent phosphorylation of 4E-BP1, but this reduction in phospho-4E-BP1 was not significantly correlated with the reduction in estimated eIF4E activity, and changes in levels of the other components had strong influences on estimated eIF4E activity. For example, 4E-BP1 expression changed considerably (mean -0.3; range -5 to +5; $p=0.01$), meaning that 8 individual decreases in phospho-4E-BP1 could be explained at least partially by reductions in total 4E-BP1, as opposed to reduced phosphorylation. 4E-BP2 expression also frequently changed (mean +2.2; range -2 to +7; $p<0.001$), while some individuals showed dramatic changes in expression of eIF4E (mean -0.1; range -6 to +4). Interestingly, changes in eIF4E and 4E-BP1 were positively associated ($r=0.60$, $p=0.003$), often resulting in relatively small changes in estimated eIF4E activity, despite substantial fluctuations in expression of the individual proteins. Critically, neither reduced estimated eIF4E activity or reduced phospho-4E-BP1 correlated with reduced Ki67.

A striking observation was that post-treatment levels of eIF4E and the 4E-BPs frequently varied considerably from pre-treatment levels. Our hypothesis was that these changes represent development of acquired resistance to inhibition of eIF4E activity, by induction of changes in eIF4E regulation within tumour cells or by clonal selection of cells with different eIF4E-related expression profiles. Importantly, this proposed acquired resistance is not necessarily reflected in higher proliferation rates. Using this hypothesis, one would predict that tumours with high pre-treatment estimated eIF4E activities would be most subject to drug-induced expression changes or to clonal selection pressures, and would show the greatest adaptive changes in eIF4E regulation. In fact, high estimated eIF4E activity pre-treatment did positively correlate with the combined magnitude of changes in expression of the four markers ($r=0.46$, $p=0.03$) and, in particular, with increases in 4E-BP2 expression ($r=0.55$, $p<0.01$). We concluded that high estimated eIF4E activity may, in fact, predict tumour response to everolimus; however this response is not the expected reduction in proliferation, but is development of changes in eIF4E regulation, presumably to promote everolimus resistance. This work has been published[11] (manuscript attached in appendices).

3. Key Research Accomplishments

1) We established that mathematically combining assessments of expressions of eIF4E-regulators with assessments of expression of eIF4E in clinical tumours provides improved prognostic insights over examination of eIF4E alone, as this combinatorial function estimates eIF4E *activity* (Coleman et al 2009).

2) We demonstrated that an experimentally determined measure of eIF4E activity predicts cellular sensitivity to rapamycin *in vitro*. However, this result was not reproduced in breast cancer patients. Estimates of eIF4E activity had little predictive value for pre-surgery treatment with rapamycin derivative everolimus. We interpret this to suggest that easily and rapidly acquired resistance may limit the worth of everolimus monotherapy as a treatment for breast cancer (Satheesha et al 2011).

4. Reportable outcomes

This work has led directly to three published papers (attached in appendices):

Coleman LJ, Peter MB, Teall TJ, Brannan RA, Hanby AM, Honarpisheh H, Shaaban AM, Smith L, Speirs V, Verghese ET, McElwaine JN, Hughes TA (2009) Combined analysis of eIF4E and 4E-binding protein expression predicts breast cancer survival and estimates eIF4E activity. Br J Cancer 100, 1393-9

Smith L, Coleman LJ, Cummings M, Satheesha S, Shaw SO, Speirs V, Hughes TA (2010) Expression of estrogen receptor beta isoforms is regulated by transcriptional and post-transcriptional mechanisms. Biochem J 429, 283-290

Satheesha S, Cookson VJ, Coleman LJ, Ingram N, Madhok B, Hanby AM, Suleman CAB, Sabine VS, Macaskill EJ, Bartlett JMS, Dixon JM, McElwaine JN, Hughes TA (2011) Response to mTOR inhibition: activity of eIF4E predicts sensitivity in cell lines and acquired changes in eIF4E regulation in breast cancer. Mol Cancer 10, 19

5. Conclusions

We tested and supported our main hypothesis: analysis of eIF4E *activity* in breast tumours gives improved prognostic insights into breast cancer over analysis of only eIF4E expression.

We also demonstrated that measurement of eIF4E activity provides a predictive marker for response of cell lines to treatment with the drug rapamycin. However, we found this not to be the case for breast cancer patients with estimates of eIF4E activity in biopsies taken at diagnosis having no predictive value for apparent response to pre-operative treatment with the rapamycin derivative everolimus. We interpret this to suggest that rapidly acquired resistance may limit the worth of everolimus monotherapy in clinical breast cancer.

Finally, we have developed a generally-applicable cross-disciplinary methodology for combining the prognostic/predictive insights from individual biomarkers into estimates of the activities of biological pathways. This approach could be applied to estimate pathway activities and therefore predict response for any molecularly-directed cancer therapy. The most obvious example is the her2 pathway that is targeted by herceptin (trastuzumab). This therapy is assigned on the basis of over-expression of the biomarker and molecular target her2. However, a significant proportion of patients fail to respond to the therapy, while it has been reported that some her2-negative tumours do respond. It is likely that an estimate of her2 activity, based on combined assessments of expression of her2 and its direct regulators, would provide an improved predictive biomarker.

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Combined analysis of eIF4E and 4E-binding protein expression predicts breast cancer survival and estimates eIF4E activity

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Increased eukaryotic translation initiation factor 4E (eIF4E) expression occurs in many cancers, and makes fundamental contributions to carcinogenesis by stimulating the expression of cancer-related genes at post-transcriptional levels. This key role is highlighted by the facts that eIF4E levels can predict prognosis, and that eIF4E is an established therapeutic target. However, eIF4E activity is a complex function of expression levels and phosphorylation statuses of eIF4E and eIF4E-binding proteins (4E-BPs). Our hypothesis was that the combined analyses of these pathway components would allow insights into eIF4E activity and its influence on cancer. We have determined expression levels of eIF4E, 4E-BP1, 4E-BP2 and phosphorylated 4E-BP1 within 424 breast tumours, and have carried out analyses to combine these and relate the product to patient survival, in order to estimate eIF4E activity. We show that this analysis gives greater prognostic insights than that of eIF4E alone. We show that eIF4E and 4E-BP expression are positively associated, and that 4E-BP2 has a stronger influence on cancer behaviour than 4E-BP1. Finally, we examine eIF4E, estimated eIF4E activity, and phosphorylated 4E-BP1 as potential predictive biomarkers for eIF4E-targeted therapies, and show that each determines selection of different patient groups. We conclude that eIF4E's influence on cancer survival is modulated substantially by 4E-BPs, and that combined pathway analyses can estimate functional eIF4E.
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The eukaryotic translation initiation factor 4E (eIF4E) has key roles in carcinogenesis (De Benedetti and Graff, 2004). eIF4E is often overexpressed in carcinoma cells as compared to equivalent normal epithelium in many tumour types including breast (Kerekatte *et al*, 1995), lung (Rosenwald *et al*, 2001) and colon (Rosenwald *et al*, 1999). The oncogenic role of this overexpression has been shown by various experimental observations; for example, forced eIF4E overexpression within many cell types leads to transformation (De Benedetti and Graff, 2004), and within transgenic mice increases incidence of multiple tumour types (Ruggero *et al*, 2004). eIF4E has at least two normal cellular functions. First, it is an essential component of the multimeric factor eIF4F, which initiates cap-dependent translation – the mechanism responsible for most protein synthesis (Gray and Wickens, 1998). eIF4E's role is to bind to mRNA caps allowing recruitment of eIF4F, and subsequently the translational machinery. The complex formed scans linearly along the 5′ untranslated region (UTR) until an initiation codon in good context is encountered, at which point further elements of translational machinery are recruited and protein synthesis starts. Second, eIF4E regulates expression of some genes by controlling nuclear export of their transcripts (Culjkovic *et al*, 2007), a function that also requires eIF4E's cap-binding activity (Culjkovic *et al*, 2005). Under most normal conditions, availability of active eIF4E is thought to be rate limiting for both functions. One might expect a general translational stimulation to result from the increased eIF4E expression in cancers, on account of enhanced mRNA cap recognition, yet effects of eIF4E overexpression are more subtle. Approximately 10% of mammalian transcripts have 5′UTRs that may form complex secondary structures that reduce the abilities of both eIF4F to bind to mRNAs and the translational machinery to scan 5′UTRs (Pesole *et al*, 2001); the result is that these transcripts are translated inefficiently (Hughes, 2007). The majority of human transcripts with these inhibitory 5′UTRs code for growth or cancer-associated proteins (Kozak, 1991). Increased eIF4E is thought to reduce the effects of 5′UTR structure by enhancing cap-recognition and scanning, therefore increasing translation of these specific oncogenic transcripts (De Benedetti and Graff, 2004). Similarly, increased eIF4E expression enhances nuclear export of a set of transcripts associated with oncogenesis (Culjkovic *et al*, 2007). As a consequence of this central role, eIF4E is an established target for cancer therapy (Smolewski, 2006; Graff *et al*, 2008).

The importance of eIF4E in cancer has been underlined by the fact that eIF4E expression levels can be used to determine prognosis. Cases in which eIF4E is highly overexpressed tend to have poor prognoses (Li *et al*, 1998). A substantial confounding factor is that eIF4E expression does not equate to eIF4E activity, thereby making interpretation of potential influences of eIF4E

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levels difficult. eIF4E activity is a complex function of eIF4E expression and expressions and activities of eIF4E-binding proteins (4E-BP1, 2 and 3) that bind to and inhibit eIF4E (Richter and Sonenberg, 2005) (Supplementary Figure S1). Activity is further regulated by phosphorylation of 4E-BP1 (and other 4E-BPs it is assumed), with only hypophosphorylated forms being able to inhibit eIF4E. Additional regulation occurs by differential phosphorylation of eIF4E itself, although there are conflicting reports as to how this influences activity (Scheper and Proud, 2002). The result is that high expression of eIF4E may not lead to high eIF4E activity if, for example, hypophosphorylated 4E-BP1 were also highly expressed. Many cancer-related signalling pathways, including PI3K and p38, converge to regulate eIF4E and 4E-BP phosphorylation; therefore, eIF4E activity seems to be a key cancer-signalling node (Polunovsky and Bitterman, 2006). Here, we have tested the hypothesis that combined analyses of expressions and phosphorylation states of eIF4E, and its regulators allows greater understanding of eIF4E activity and its influence on cancer than examination of eIF4E expression alone.

MATERIALS AND METHODS

Patients

Ethical approval was obtained (Leeds East 05/Q1206/136). Archival cancer tissue and data were obtained for 424 patients diagnosed at LTH NHS Trust from 1983–2006. Tissue microarrays (TMAs) were constructed containing 0.6 mm cores selected from representative tumour areas as determined by a consultant breast histopathologist (AMS) from H&E stained sections. Survival periods – overall: initial diagnosis to death; disease-free: initial diagnosis to the diagnosis of recurrence/metastasis; disease-specific: initial diagnosis to death after recurrence or metastasis (cancer-specific death confirmed in most cases).

Westerns and immunohistochemistry

MCF7 and MDA-MB-231 cells were cultured/transfected as earlier (Johnson *et al*, 2008; Maraqa *et al*, 2008). An eIF4E expression vector was obtained from John Blenis (Harvard Medical School) and Nahum Sonenberg (McGill). Western analyses were carried out as earlier (Maraqa *et al*, 2008) using the reagents in Supplementary Table S1. TMA sections of 5 μm were dewaxed and blocked in hydrogen peroxidase block (20 min). Antigens were retrieved and stained as described in Supplementary Table S1, and as used elsewhere (Zhou *et al*, 2004; Dutton *et al*, 2005; Lee *et al*, 2005; Engelman *et al*, 2008). Envision detection was used (DAKO, Glostrup, Denmark). Negative controls (primary antibodies omitted) were included in each immunohistochemistry (IHC) batch; in addition, adjacent normal epithelium, lymphocytes and blood vessel endothelium served as internal controls. Controls were performed for p4E-BP1 antibodies in which sections were pretreated with Lambda Phosphatase (Nebraska, NE, USA). Cores were scored for immunoreactivity by two or more individuals (LJC, TJT, ETV and RAB), taking into account the average intensity and percentage of positively stained tumour cells (as used earlier for eIF4E (Zhou *et al*, 2006)). Staining intensity scores (0 no staining, 1 weak, 2 moderate and 3 strong) were added to percentages positively stained scores (1 <5%; 2, 6–25%, 3, 26–75% and 4 >75%), giving totals of 0 or 2–7. Consensus scores were determined for cores with different initial scores, and all scoring was overseen by a consultant breast histopathologist (AMH).

Mathematical analyses

Data were analysed using Kaplan–Meier survival curves. Dependence on prognostic indicators was determined using Cox proportional hazards models; significance values relate to likelihood ratio tests of the null hypothesis that indicators do not effect hazard rates (Cox and Oakes, 1984). SPSS (SPSS, Chicago, IL, USA) and the Statistics Toolbox in MATLAB (ecdf.m and coxphfit.m) (MathWorks, Natick, MA, USA) were used. Tests were two sided and $P<0.05$ was considered significant.

RESULTS

Antibody validation

We have used IHC to determine expression levels in breast tumours of the main regulatory molecules of the eIF4E pathway – namely, eIF4E, 4E-BP1, 4E-BP2 and phosphorylated 4E-BP1 (Thr37/46) (termed p4E-BP1). We have not examined 4E-BP3 because it is not thought to have a role in breast (Poulin *et al*, 1998), or phosphorylated forms of eIF4E, as their influences on the activity and in cancer remain uncertain (Scheper and Proud, 2002; Salehi and Mashayekhi, 2006; Buxade *et al*, 2008). First, we optimised the antibody use on archival breast tissue. We established that antibodies were specific for their antigens using western blots against lysates of breast cancer cell lines (Supplementary Figure S2A). In addition, we showed phospho-specificity of antibodies against p4E-BP1 by carrying out IHC on serial tissue sections with and without pretreatment with protein phosphatase (Supplementary Figure S2B).

Patient cohort and immunohistochemistry

Tissue micro-arrays containing samples from 424 breast tumours were established, supported by detailed clinicopathological data (Supplementary Table S2). The cohort included a wide range of patient and tumour characteristics, with mean patient follow-up of 91.9 months. We carried out IHC for eIF4E, 4E-BP1, 4E-BP2 and p4E-BP1 on TMA sections and assessed immunoreactivity within tumour cells, taking into account the proportions of cells staining positively and average intensity, giving scores of 0 (negative) or 2–7 (positive). Representative staining patterns are shown (Figure 1, and at higher magnification in Supplementary Figure S3). Tumour stroma and normal tissue were negative for eIF4E, 4E-BP1 and 4E-BP2, whereas very occasional low-intensity staining for p4E-BP1 was noted in normal epithelial cells. Staining was generally cytoplasmic, although nuclear staining was noted in a minority of cases (Supplementary Figure S4); this was separately analysed and was found not to be of prognostic value and is not discussed. As expected, data were not available for some patients because of the TMA core loss during processing, a well-recognised occurrence, therefore, data for all four antigens were available for only 282 patients. The full range of scores were observed for each antigen (Figure 1I). It was notable that staining was most frequently not detectable for p4E-BP1. Others have reported more frequent expression of p4E-BP1 (Zhou *et al*, 2004), therefore we carried out IHC for an alternative p4E-BP1 species (Ser65); we found immunoreactivity with this antibody to be similarly infrequent (see discussion).

Expressions of eIF4E and 4E-BPs correlate with grade

Associations between antigen expressions and a wide range of clinicopathological parameters were examined. No correlations were found with nodal status, tumour size or histological type. Weak positive/borderline no correlations were found with oestrogen receptor α status and eIF4E expression (Spearman's ρ coefficient 0.21; $P<0.001$) and 4E-BP2 (0.22; $P<0.001$), but not with 4E-BP1 or p4E-BP1. Strong correlations between expression of markers and tumour grade were found. eIF4E expression (split into three classes, 0–3, 4–5 and 6–7) was positively associated with grade (χ^2-test, $P=0.011$), whereas expression of both 4E-BPs was negatively associated with grade

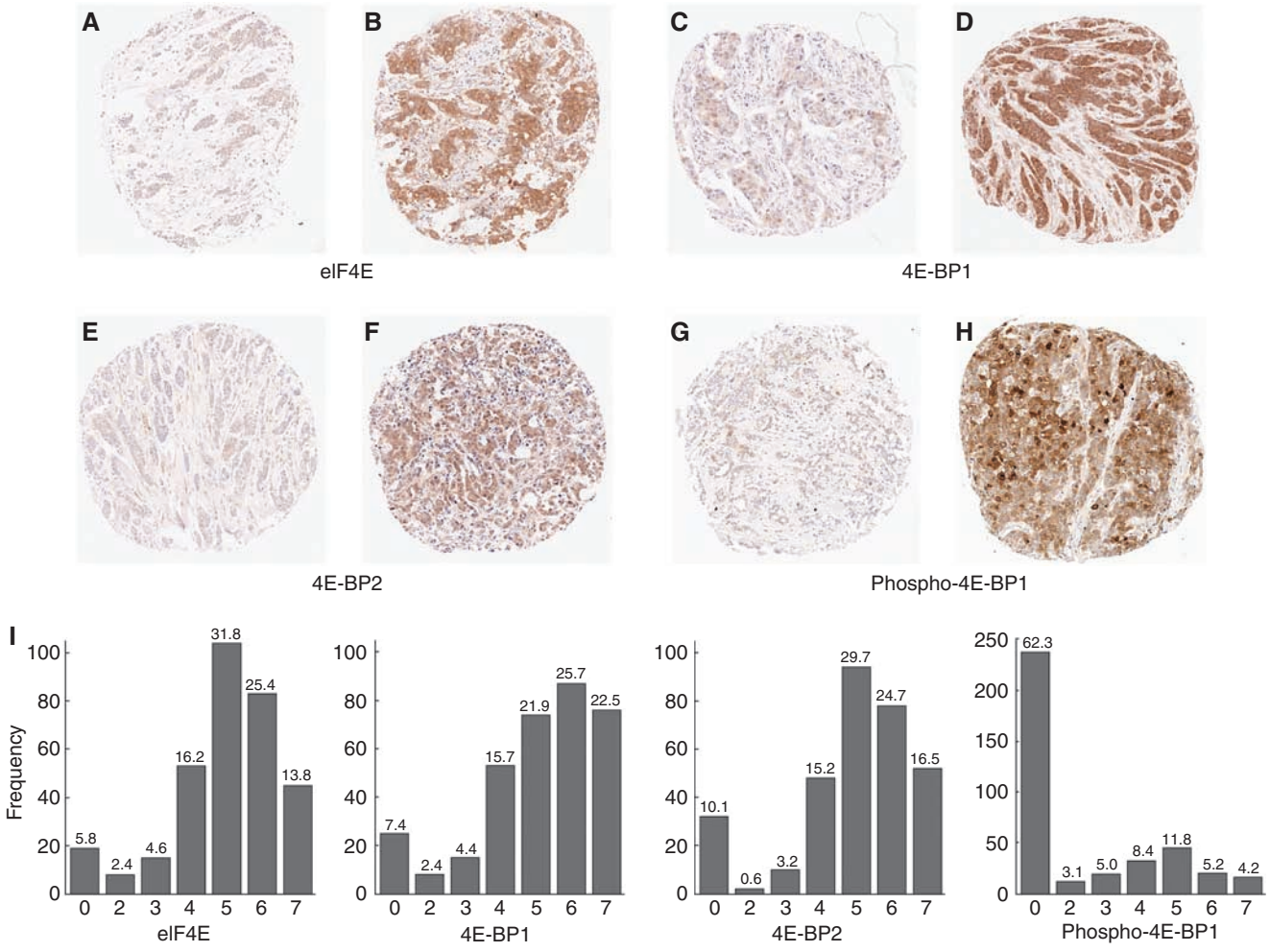

Figure 1 The full range of expression intensities and proportions for eukaryotic translation initiation factor 4E (eIF4E), eIF4E-binding protein (4E-BP) 1, 4E-BP2 and p4E-BP1 occur within breast cancers. (**A**–**H**) Representative tumour tissue microarray cores showing immunoreactivity as labelled. These cores were scored A 4, B 7, C 3, D 7, E 3, F 7, G 3 and H 7. (**I**) Histograms showing distributions of immunohistochemistry scores within breast cancers. Scores (x-axis) and numbers of cores assigned to each score (y-axis) are shown. Percentages of the cohort are given above the bar for each score.

(4E-BP1 $P=0.002$; 4E-BP2 $P=0.029$). p4E-BP1 was positively associated with grade ($P=0.012$). A positive association between eIF4E expression and grade has been reported earlier (Li *et al*, 2002). Correlations for other markers were consistent with their influences on carcinogenesis being through the eIF4E pathway; 4E-BPs, eIF4E inhibitors, were negatively associated with grade, whereas 4E-BP1 phosphorylation, which would relieve 4E-BP1-induced inhibition of eIF4E, was positively associated.

High expression of eIF4E correlates with poor prognosis

Kaplan–Meier survival analyses were used to determine survival with respect to eIF4E. Analyses were carried out with expression divided into IHC scores, although scores of 0, 2 and 3 were combined as each individual group was small, for overall survival (OS), disease-free survival (DFS) and disease-specific survival (DSS) (Figure 2A–C). High eIF4E scores were indicative of poor prognosis. Prognosis seemed to worsen with each increasing eIF4E score for OS, whereas patterns for DFS and DSS suggested weaker, but still detectable, influences of individual scores with an overall grouping into two classes (0–5 good prognosis; 6 or 7 poor prognosis). We have examined relationships between eIF4E expression and survival using Cox regressions. We have included either eIF4E scores, or eIF4E expression dichotomised arbitrarily or as suggested by the apparent bimodal distribution seen above, and modelled these with respect to OS, DFS and DSS. Models that most accurately reflected the data included eIF4E scores rather than dichotomised data, showing the value of scoring proportion and intensity of positive tumour cells. In these models each increase of 1 in eIF4E score gave increases in hazard ratios (HRs) of 1.22 ($P=0.004$), 1.3 ($P=0.008$) and 1.33 ($P=0.005$) for OS, DFS and DSS, respectively. Thus individuals with scores of 7 have DFS HRs of 6.15 (95% CIs: 3–12, $P=0.008$) as compared with individuals with scores of 0. The prognostic value of eIF4E has been reported earlier as independent of grade/nodal status in breast cancer (Li *et al*, 1998); we have examined independence from the Nottingham Prognostic Index (NPI), which takes account of tumour size, grade and lymph node status (Haybittle *et al*, 1982). In multivariate Cox regressions the prognostic value of eIF4E was independent of NPI with eIF4E remaining significant for OS (NPI $P<0.00001$; eIF4E $P=0.02$), DFS (NPI $P<0.00001$; eIF4E $P=0.045$) and DSS (NPI $P<0.00001$; eIF4E $P=0.029$).

Prognostic value of 4E-BP1, 4E-BP2 and p4E-BP1

Kaplan–Meier survival analyses were also used to determine survival with respect to the other markers. We present data

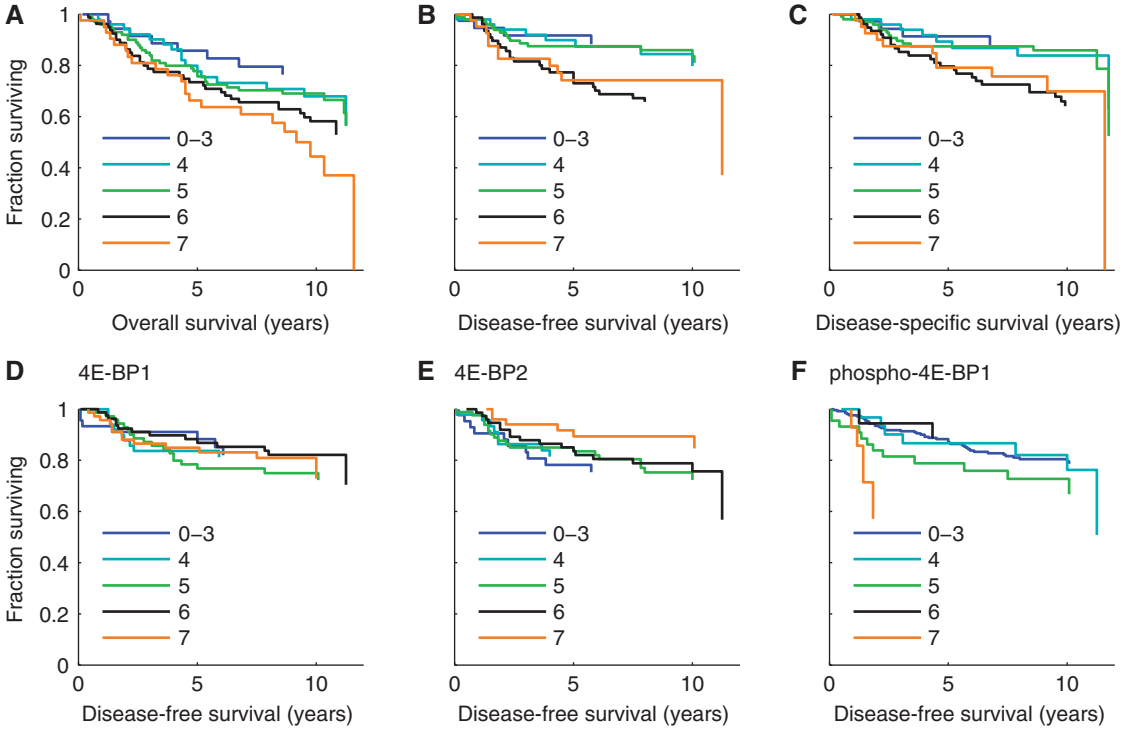

Figure 2 Expression of eukaryotic translation initiation factor 4E (eIF4E), but not eIF4E-binding protein (4E-BP) 1, 4E-BP2 or p4E-BP1, is associated with prognosis. (**A**–**C**) Kaplan–Meier survival analyses for overall (**A**), disease-free (**B**) and disease-specific survival (**C**) for patient groups with tumours with differing eIF4E scores. (**D**–**F**) Kaplan–Meier survival analyses for disease-free survival for patient groups with tumours with differing scores for 4E-BP1 (**D**), 4E-BP2 (**E**) or p4E-BP1 (**F**). As scores of 0, 2 and 3 were relatively rare, these have been grouped together. Censoring ticks have been omitted for clarity.

for DFS (Figure 2D–F), and for OS and DSS (Supplementary Figure S5). These antigens provided little prognostic insight and we were unable to construct significant Cox equations to model their individual survival influences. In the case of 4E-BP2, there was a nonsignificant trend for high scores to associate with good prognosis.

Mathematical modelling of influences of 4E-BPs: 4E-BPs modify eIF4E activity and provide additional prognostic insights

Next we examined influences of 4E-BPs in the context of eIF4E expression. We necessarily restricted these analyses to the 282 patients for whom scores of all four antigens were available. Using this dataset, models including solely eIF4E expression gave HRs of 1.21 ($P=0.011$), 1.24 ($P=0.035$) 1.27 ($P=0.02$) for OS, DFS and DSS, respectively, for each increase of 1 in eIF4E score. We were unable to construct significant Cox models on the basis of combinations of 4E-BPs without including eIF4E, suggesting that eIF4E is their critical effector. We found models combining expression of 4E-BP1 or p4E-BP1 (as IHC scores, or dichotomised into two groups) with eIF4E, provided little additional prognostic value over that found with eIF4E alone. However, including 4E-BP2 in a model for OS enhanced the model significantly with hazard increasing by 1.28 with each point increase in eIF4E score ($P=0.005$) and decreasing 0.11 with each increase in 4E-BP2 score ($P=0.02$). We refined this by combining eIF4E and 4E-BP2 scores into a single non-linear variable in which high levels of eIF4E or 4E-BP2 act to increase or decrease the value respectively (achieved using max (0,X-B2/3.5), where X and B2 represent eIF4E and 4E-BP2 scores). This variable, termed '*y*', predicted survival more accurately than examination of eIF4E alone; each increase of 1 in *y* carried HRs of 1.32 ($P=0.0003$), 1.32 ($P=0.013$) and 1.36 ($P=0.006$) for OS, DFS and DSS, respectively. We also investigated Cox models including expression of all four antigens. In order to combine terms, we considered models that included each variable separately and found their relative effect on HRs using maximum likelihood estimation. Although individual components were not statistically significant and had only little effects on likelihoods (with the exception of eIF4E), a combination gave improved prognostic power. This variable termed '*z*' can be described as X–B1/4+PB1/2-B2/4, where B1 and PB1 represent 4E-BP1 and p4E-BP1. Each increase of 1 in *z* gave HRs of 1.15 ($P=0.006$), 1.26 ($P=0.002$) and 1.28 ($P=0.0008$) for OS, DFS and DSS, respectively. This variable has a highly significant relationship with survival but this should be treated with caution because the constants were determined using regressions for OS, and, thus to an extent, significance is self fulfilling, at least for OS. The utility of *z*, however, is supported by the fact that its relationship with survival is more significant with DFS and DSS than OS, a result not predetermined by the approach. We also examined whether *y* or *z* give prognostic insights independently of NPI using multivariate analyses. NPI and either *y* or *z* remain significant in models for DFS (NPI $P<0.00001$; *y* $P=0.04$ or *z* $P=0.03$) and DSS (NPI $P<0.00001$; *y* $P=0.02$ or *z* $P=0.02$).

The statistical significance of relationships of *y* and *z* with survival show additional prognostic value from examining multiple eIF4E pathway components. In addition, we have shown the value of these variables using Kaplan–Meier analyses. First, we focused on patients with high eIF4E scores (6 or 7), as it is in this context that differential expression of 4E-BPs would be most relevant. Patients with eIF4E scores 6 or 7 have a relatively poor prognosis (Figure 2A–C), but no difference was detected between groups scored as 6 or 7 in terms of DFS (Figures 2B and 3A). When *y* was applied to this cohort some discrimination occurred with improved prognosis for patients whose *y* scores were lowered by 4E-BP2 (Figure 3B), although the discrimination remained statistically nonsignificant. When *z* was applied to this cohort

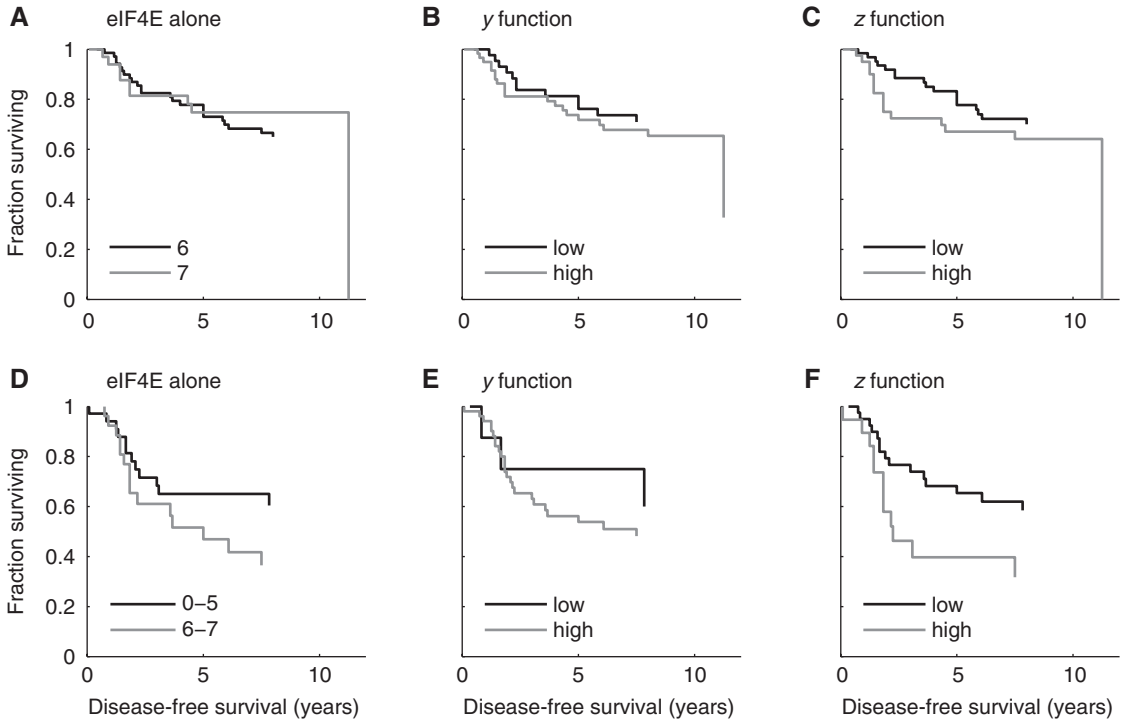

Figure 3 Additional prognostic information is gained by combining assessment of eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs) with eIF4E analysis. Kaplan–Meier survival analyses of disease-free survival for patients with either high (6 or 7) eIF4E scores (**A**–**C**) or high (>5.4) Nottingham Prognostic Index scores (**D**–**F**). Patients were dichotomised using either eIF4E expression (**A** and **D**), the *y* function (which includes eIF4E and 4E-BP2 scores) (**B** and **E**), or the *z* function (which includes scores for all 4 markers) (**C** and **F**).

further discrimination occurred (Figure 3C) showing how 4E-BPs affect patient outcome through eIF4E. Second, we have focused on patients with high NPI (and consequently poor DFS, Supplementary Figure S6). These patients were further stratified according to eIF4E expression (cutoff 5.5 as suggested by the distribution in Figure 2B) into separate groups (Figure 3D). As before, when *z* was applied (Figure 3F) further discrimination occurred allowing identification of patients with very poor (high *z*), or relatively good prognosis (low *z*). In this case, *z* discriminated into statistically significantly different groups (Figure 3F Log rank $P=0.039$) when use of eIF4E alone was not significant (Figure 3D Log rank $P=0.15$). In this case *y* was substantially less successful as a prognostic indicator (Figure 3E Log rank $P=0.5$) showing the importance of combining all four components.

Expressions of eIF4E and 4E-BPs are positively associated

Although including assessments of 4E-BPs provided additional prognostic information over that from only eIF4E, we were surprised that influences of 4E-BPs, especially of 4E-BP1, were relatively weak. One explanation for this was that expression levels of eIF4E and 4E-BPs were not independent of each other. This would mean that that at a given eIF4E level, differential expression of 4E-BPs – therefore differential modification of eIF4E activity – would be relatively rare, thereby minimising apparent influences of 4E-BPs in our analyses. Associations between marker expressions were examined using Spearman's correlation tests (Table 1). eIF4E expression showed moderate positive associations with expression of both 4E-BPs ($P<0.0001$), although weak positive/no association with p4E-BP1. Expression of 4E-BPs was also moderately positively associated with each other ($P=0.02$). Expression of p4E-BP1 was positively associated with 4E-BP1 ($P<0.0001$) (expected as 4E-BP1 must be expressed to be phosphorylated).

Table 1 Expressions of eIF4E, 4E-BP1 and 4E-BP2 are positively associated

	eIF4E	4E-BP1	4E-BP2	p4E-BP1
eIF4E	—	0.31	0.34	0.21
4E-BP1	0.31	—	0.37*	0.36
4E-BP2	0.34	0.37*	—	0.14
P4E-BP1	0.21	0.36	0.14	—

Abbreviations: eIF4E = eukaryotic translation initiation factor 4E; 4E-BP = eIF4E-binding protein; P4E-BP1 = phosphorylated 4E-BP1. Spearman's ρ correlation figures are shown. Associations are either moderately (*$P=0.02$) or highly significant (others $P<0.0001$).

eIF4E activity scores are potential biomarkers for eIF4E-targeted therapies

The eIF4E pathway is a target for cancer therapy with drugs that either inhibit eIF4E activity indirectly (mTOR inhibitors that reduce 4E-BP1 phosphorylation thereby inhibiting eIF4E (Smolewski, 2006)), or directly (by binding to/reducing expression of eIF4E (Graff *et al*, 2008)). However, a concern with these agents is toxicity resulting from general translational repression. Consequently, selection of patients who are most likely to benefit from such agents using predictive biomarkers may aid their efficacy. Three selection criteria are apparent: individuals with highest eIF4E levels, highest eIF4E activities or highest levels of phosphorylated 4E-BP1 (especially relevant for mTOR inhibitors as these act by reducing 4E-BP phosphorylation). We have compared selection of potential treatment groups using these criteria from our cohort for whom scores of all four antigens were available: first (group 1), those with eIF4E scores of 7 (37/282; 13.1%); second (group 2), those with high estimated pathway activities ($z\geqslant 5.75$, cutoff chosen to give a similar-sized group,

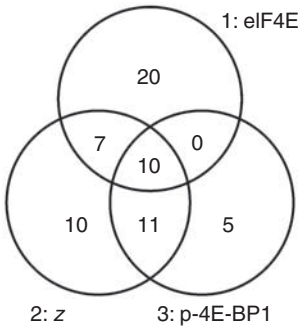

Figure 4 Use of biomarkers for dividing patients into potential treatment groups for eukaryotic translation initiation factor 4E (eIF4E)-directed therapy; different markers select very different groups. A Venn diagram demonstrating relationships between potential treatment groups selected on the basis of high eIF4E expression (1), high estimated eIF4E activity ('*z*') (2), and high p4E-BP1 expression (3).

38/282; 13.5%); finally (group 3), those with p4E-BP1 scores of 6 or 7 (26/282; 9.2%). We have examined whether biomarker choice leads to selection of different potential treatment groups (Figure 4). Substantially different patient groups were selected using each potential predictive biomarker. Only 10 patients appeared in all groups (16% of total patients selected in any group). Group 1 contained the largest proportion of uniquely selected individuals (56% of total), whereas the majorities of groups 2 and 3 (74 and 81% respectively) overlapped with at least one other group. Group 2 (high *z* values) had large overlaps with both other groups (46% of group 1 and 81% of group 3). In addition, group 2 included all individuals with high eIF4E *and* p4E-BP1 (i.e. individuals likely to have high eIF4E activity by all measures) reflecting the fact that *z* successfully takes account of both eIF4E and p4E-BP1, thereby supporting its utility as a potential predictive marker.

DISCUSSION

Expression of eIF4E in cancer has been studied extensively, however, expression does not equate to activity; therefore, interpretation of its influence is more complex than simply assessing expression. Our hypothesis was that combined examination of eIF4E and its regulators would allow greater insights into eIF4E's influence on cancer. Therefore, we determined the expression levels of eIF4E and its most well-established regulatory proteins 4E-BP1, 4E-BP2 and p4E-BP1 within tumour cells of a large cohort of cancer patients, and have combined these data into an improved measure of prognosis and estimate of eIF4E activity. In common with initial publications on eIF4E's role in cancer (Kerekatte *et al*, 1995; Li *et al*, 1998) and much of the subsequent literature, we have focused on breast cancer.

There is a wealth of literature on eIF4E expression in cancer and, although patient cohorts used have been relatively small (<200 individuals), the conclusion that high eIF4E levels are associated with poor prognosis is well established (De Benedetti and Graff, 2004). We have also found this (Figure 2A–C) using the largest cohort to date. The 4E-BPs have received less attention. 4E-BP1 is more highly expressed in tumours than normal tissues (Salehi and Mashayekhi, 2006), and expression correlates inversely with tumour progression (Martin *et al*, 2000) – observations in agreement with our findings. Despite the inverse correlation with progression, we found examination of 4E-BP1 expression not to give significant prognostic insights (Figure 2D, Supplementary Figure S5A). To our knowledge, there are no published studies concerning 4E-BP2 in cancer samples. We found that 4E-BP2 showed a nonsignificant trend for association with good prognosis (Figure 2E, Supplementary Figure S5B). High p4E-BP1 levels have earlier been shown to correlate with grade and poor prognosis (Zhou *et al*, 2004; Castellvi *et al*, 2006; Rojo *et al*, 2007). In contrast, we found detectable p4E-BP1 (Thr37/46) in only 37.7% of the patients compared with $>59\%$ in other reports (Zhou *et al*, 2004; Rojo *et al*, 2007; Akcakanat *et al*, 2008)), and to provide little prognostic power when analysed alone (Figure 2F, Supplementary Figure S5C). We have carried out additional analyses with the same antibody as reported earlier for p4E-BP1 (Ser65) (Zhou *et al*, 2004) and similarly found low expression (50% undetectable, 28% the lowest positive score). These differences may relate to the cohorts used as earlier studies had higher proportions of node positive (Zhou *et al*, 2004), subsequently metastatic (Akcakanat *et al*, 2008) or high-grade cases (Rojo *et al*, 2007).

There are very few studies where multiple eIF4E pathway components have been analysed. The ratios of p4E-BP1 to total 4E-BP1 and of eIF4E to 4E-BP1 have been shown to correlate with high tumour grade (Salehi and Mashayekhi, 2006; Armengol *et al*, 2007), but their relationships with survival were not examined. A positive correlation between expression of eIF4E and p4E-BP1 has also been noted, with the conclusion being that eIF4E was 'active' in these cells (Nathan *et al*, 2004). We have determined expression levels of eIF4E, 4E-BP1, 4E-BP2, p4E-BP1 and have undertaken analyses to relate these together and to survival. First, we found no statistically significant relationship to survival for any combination without including eIF4E – suggesting that eIF4E is the critical effector. Second, in support of our initial hypothesis, we showed that combined assessment of the four components allowed improved prognostic insights over eIF4E alone ('*z*' function; text, Figure 3). It is important to note that we did not predetermine relationships between components in *z*, rather these were defined by the best fit with the data; the fact that the relationships reflect our expectations from understanding the pathway supports the view that *z* is a true estimate of eIF4E activity. Third, we showed differential expression of 4E-BP2 in cancer to be more influential in terms of survival than 4E-BP1. This was shown by the observations that expression of 4E-BP2, but not 4E-BP1, showed a trend towards being a prognostic factor alone (Figure 2E, Supplementary Figure S5B), provided an improved prognostic indicator in combination with eIF4E (*y*), and was the most statistically significant component of *z* after eIF4E itself. This observation may relate to the fact that 4E-BP2 binds, and therefore inhibits eIF4E more strongly than 4E-BP1 (Abiko *et al*, 2007). Interestingly, we found that expressions of eIF4E and 4E-BPs were positively associated (Table 1): an unexpected finding as they are functionally opposed and correlate oppositely with grade. One explanation is that 4E-BP translation may be specifically derepressed by eIF4E's action on the 5′UTRs of their transcripts, representing a negative feedback loop within the eIF4E pathway.

Clinical trials of the efficacy and safety of cancer therapeutics that target eIF4E have been carried out (Graff *et al*, 2008) and some toxicity has been reported (O'Donnell *et al*, 2008; Tabernero *et al*, 2008). Selection of individuals who are most likely to benefit from the agents may be appropriate in order to avoid potentially harmful and/or ineffective therapy in some patients. We show that substantially different patient groups are chosen using three potential predictive biomarkers, and therefore that use of the best biomarker is important for targeting of these therapies (Figure 4). Patients with high estimated eIF4E activity ('*z*') (group 2) and high p4E-BP1 levels (group 3) should provide good candidates for treatment. The former group has a particularly poor prognosis (Figure 3C and F), and therefore great potential for clinical benefit from these drugs.

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online data

Expression of oestrogen receptor β isoforms is regulated by transcriptional and post-transcriptional mechanisms

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Although ERs (oestrogen receptors) mediate breast tumour behaviour, the precise role of ERβ remains unclear. This is mainly because analyses have been complicated by the presence in breast tissue of three ERβ protein variants (ERβ1, ERβ2 and ERβ5) that derive from differential 3′ splicing. We have recently identified the first known mechanisms responsible for the differential control of isoform expression, involving regulation of translation via 5′-UTRs (untranslated regions). In the present study, we have uncovered further complexity involving the influence of multiple promoters and cross-talk between 5′- and 3′-UTRs. We demonstrate that full-length ERβ mRNAs are transcribed from three separate promoters; two promoters are well-established within the literature, whereas the third represents a novel finding. Each promoter produces transcripts with distinct 5′-UTRs. The differential 3′ splicing that produces transcripts coding for the ERβ isoforms also defines isoform-specific 3′-UTRs. We identified exact 3′-UTR sequences for each isoform, and have shown that alternative polyadenylation sites are used in a cell-type specific manner to produce transcripts with 3′-UTRs of different lengths. Critically, we show that 5′- and 3′-UTRs combine to specify the efficiencies with which individual transcripts are translated, with 3′-UTR length having a key influence. In addition, we demonstrate how 17β-oestradiol, a key driver of breast cancer development, affects the regulation of ERβ expression at both transcriptional and translational levels.

Key words: breast tissue, differential polyadenylation, *esr2*, oestrogen receptor (ER), translational regulation, untranslated region (UTR).

INTRODUCTION

ERs (oestrogen receptors) are critical mediators of oestrogen function and play roles in many pathological processes, especially carcinogenesis [1]. Although the roles of ERα are relatively well understood, those of ERβ remain unclear. This is due, in part, to a reported discrepancy between ERβ expression at mRNA and protein levels, leading to conflicting expression data [2,3]. Also, ERβ is expressed as at least five protein isoforms, derived from differential 3′ splicing of ERβ transcripts [4], yet their potential to have distinct functions has often been ignored in favour of analysis of total ERβ. In breast tissue, ERβ1, ERβ2 and ERβ5 predominate, with each appearing to have separate biological functions as demonstrated by their associations with different breast cancer types and prognoses [5–8]. Differences between the isoforms are also evident in terms of comparisons of expression in normal breast tissue and breast cancer. ERβ1 is frequently down-regulated in cancer compared with normal cells [9,10], suggesting that it may function as a tumour suppressor [11–13]. However, ERβ2 appears to be up-regulated during carcinogenesis [10,14], whereas ERβ5 may also be up-regulated, at least at the level of mRNA [15]. Little is known about the mechanisms responsible for these changes in ERβ expression. ERβ promoter methylation has frequently been observed in breast cancers, and this is thought to be responsible for down-regulation of some ERβ transcripts, although this appears to be at odds with the reported up-regulation of ERβ2 and ERβ5. We have recently identified the first known mechanisms responsible for differential control of expression of the different isoforms involving regulation of translation via two alternative 5′-UTRs (untranslated regions) [16]. We have now extended this theme to examine thoroughly the regulatory functions of the extensive range of ERβ UTRs, and have determined further mechanisms for differential control of ERβ isoform expression.

EXPERIMENTAL

Cell culture, transfection, flow cytometry and dual luciferase assays

Cell lines representing breast cancers of luminal (MCF7) and basal (MDAMB-231) subtypes and benign non-transformed breast tissue (HB2) were obtained from the European Collection of Animal Cell Cultures. Cells were cultured in RPMI 1640 medium containing 5 % (v/v) FBS (fetal bovine serum) (MCF7, MDAMB-231) or DMEM (Dulbecco's modified Eagle's medium) containing or 10 % (v/v) FBS (HB2) (both Invitrogen) at 37 °C under 5 % CO_2. Bi-monthly mycoplasma checks (MycoAlert® Mycoplasma detection assay, Lonza) were consistently negative and STR (short tandem repeat) profiles confirmed cell identity. Cells were transfected as described previously [16]. For experiments using exogenous E2 (17β-oestradiol), cells were cultured in Phenol Red-free medium (Invitrogen) supplemented with charcoal-stripped FBS. At 5 h post-transfection, fresh medium or medium containing 10 nM E2 (Sigma–Aldrich) was added before analysis after 24 h. For flow cytometry, cells were removed from wells with trypsin and resuspended in fresh medium containing 1 % (v/v) serum. GFP (green fluorescent protein) expression was quantified (mean fluorescent intensity of 10^4 events after exclusion of debris/dead cells on the basis of forward activated light scatter against side scatter) at 525 nm (LSRII, BD Biosciences). Gates were set so that <1 % of untransfected

Abbreviations used: E2, 17β-oestradiol; ER, oestrogen receptor; FBS, fetal bovine serum; GFP, green fluorescent protein; ORF, open reading frame; qPCR, quantitative PCR; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; uORF, upstream ORF; UTR, untranslated region.

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cells were defined as expressing GFP. Dual-luciferase assays (Promega) were performed according to the manufacturer's instructions using a Lumat LB9507 luminometer (Berthold Technologies). pSV40-*Renilla* (Promega) was used as a control.

Plasmid construction

pTH-GFPa and GFP reporters for UTRa and UTRc have been described previously [16,17]. The GFP reporter for the E1 5′-UTR was cloned similarly to those for UTRa and UTRc; the UTR was amplified by PCR from cDNA prepared from MCF7 cells and was cloned upstream of the GFP ORF (open reading frame) in pTH-GFPa. Importantly, this strategy removes the 5′ end of the multiple cloning site, allowing the inserted 5′-UTRs to be immediately adjacent to the transcriptional start site. 3′-UTRs were amplified by PCR from cDNA prepared from HB2 cells and were cloned downstream of the GFP ORF in each of the 5′-UTR reporter constructs with BamHI/HindIII. Promoter sequences were amplified from MCF7 genomic DNA and cloned into pGL3-Basic (Promega) using KpnI/NheI. The E1 promoter was cloned as two fragments: a 3′ fragment cloned using KpnI/NheI, then a 5′ fragment cloned using KpnI. Primer sequences are listed in Supplementary Table S1 at http://www.BiochemJ.org/bj/429/bj4290283add.htm.

cDNA synthesis and PCR

RNA was purified from cells with RNeasy kits (Qiagen); contaminating DNA was removed with Turbo DNase I (Applied Biosystems). First-strand cDNA was synthesized using SuperScript II (according to the manufacturer's protocol) and oligo(dT) or random hexamers. Triplicate real-time PCR analysis was performed (Applied Biosystems SYBR® Green PCR Master Mix and 7900HT machine). Dissociation curves and serial cDNA dilutions were performed to ensure primer specificities and equivalent amplification efficiencies; correlation coefficients of >0.985 and primer efficiencies of $>95\,\%$ and $<100\,\%$ were deemed acceptable. Reactions were also performed using template lacking RT (reverse transcriptase): products were either undetectable or greatly reduced (>30000-fold less product than the equivalent RT+), hence genomic or plasmid DNA contamination was not considered to interfere with data. Expression of UTRs was determined relative to expression of the *RPLP0* (36B4) gene [18]. RACE (rapid amplification of cDNA ends) was performed using 5′RACE System 2 or 3′RACE System (both Invitrogen) according to the manufacturer's instructions (both standard and a modified adapter primer were used for ERβ2 3′RACE). All primer sequences are listed in Supplementary Table S1. Products were analysed on 2.5 % agarose gels [0.5 μg/ml ethidium bromide, 1 × TBE (45 mM Tris/borate and 1 mM EDTA)] and visualized on an UV transilluminator. Products were excised from gels and cloned into pGEM-Teasy (Promega); at least five clones for each were sequenced. Note that products are larger than the UTRs they represent since they include some reading frame and the RACE adapters.

RNA structure and statistical analyses

Modelling was performed using mfold v3.1 to predict potential secondary structures for RNA molecules as described previously [16]. The algorithm finds base-pairing solutions that are sterically possible and release the greatest amount of free energy (ΔG) during structural folding; more stable structures release more energy as they form and therefore have greater ΔG values [19]. Student's t test was used for statistical analysis using Microsoft Excel. All P values were two-sided; $P < 0.05$ was considered significant.

RESULTS

A novel 5′-UTR for ERβ

We studied previously the regulatory roles of two ERβ 5′-UTRs that we termed UTRa and UTRc [16]. These 5′-UTRs result from transcription initiation from two alternative promoters and mutually exclusive splicing of the untranslated first exons, exon 0K or 0N, to the first coding exon (exon 1) [20]. 5′-RACE analyses were performed in breast cell lines in order to examine ERβ 5′-UTRs further. Products representing 5′-UTRs containing only sequence from exon 1 or from immediately upstream of the 5′ splice site of exon 1 were the sole products obtained from primers located within the coding region of exon 1 (Figure 1A). UTRa or UTRc could only be detected by 5′-RACE using primers specific for their respective upstream exons, 0K and 0N [16]. RACE products containing only sequence from within the accepted bounds of exon 1 could be interpreted in two ways: as truncated versions of UTRa or UTRc, or as complete 5′-UTRs derived from transcriptional initiation within exon 1. Products containing sequence upstream of the accepted bounds of exon 1 were likely to result from transcriptional initiation adjacent to exon 1. Figure 1(B) shows an alignment of mRNAs containing UTRa, UTRc or the novel shorter 5′-UTR ('UTR-E1') with the 5′ end of the human ERβ gene (*esr2*) on 14q23. The diagram depicts putative transcriptional initiation (bent black arrows) over a range of sequences upstream of and within exon 1 allowing the expression of a 5′ extension to exon 1 (grey box) on some UTR-E1-containing transcripts. We next performed qPCR (quantitative PCR) to examine the relative expression of UTRa, UTRc and UTR-E1 in breast cell lines. We analysed expression of UTR-E1 using a primer that was complementary to the sequence within this 5′ extension of exon 1, and was therefore not contained within UTRa or UTRc. It is worth noting that these analyses may under-represent UTR-E1 expression, since RACE analyses show that the 5′ extension of exon 1 is present on only a subset of UTR-E1-containing transcripts. All three breast cell lines examined expressed UTR-E1 (Figure 1C). Products were not amplified from mock reverse transcription reactions, providing validation that transcribed/reverse-transcribed sequences were detected rather than contaminating genomic DNA. In HB2 and MCF7 cells, UTRa was the majority species, with UTRc and UTR-E1 being expressed at similar lesser levels. In contrast, MDAMB-231 cells expressed $\sim$12.5-fold more UTRc, and $\sim$3.5-fold more UTR-E1, than UTRa. We concluded that UTR-E1 represented a novel 5′-UTR for ERβ.

A third transcriptional promoter for ERβ

Next, our hypothesis was that the DNA immediately upstream of exon 1 acts as a promoter allowing expression of transcripts containing UTR-E1. We cloned $\sim$2 kb of the genomic DNA directly upstream of exons 0K, 0N or E1 into separate luciferase reporter vectors and performed luciferase assays in HB2 and MCF7 breast cell lines in order to examine the relative promoter activities of the two known ERβ promoters and the putative third promoter. Activities are shown in each cell type (Figure 2). The putative third promoter ('promoter E1') showed activity in both cell lines. In HB2 cells, promoter E1 showed intermediate activity between promoters 0N, the most active, and 0K, the least active. In MCF7 cells, promoters 0K and 0N determined similar transcriptional activities, whereas promoter E1 was $\sim$70 % weaker. We concluded that ERβ transcription is indeed driven

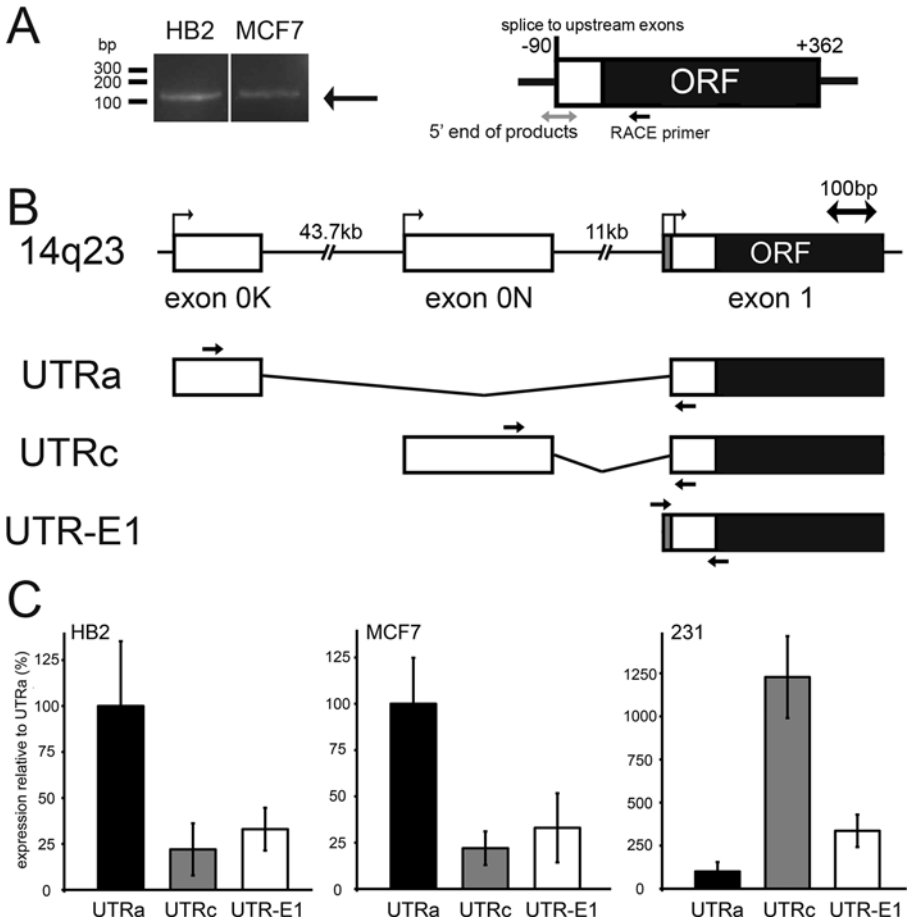

Figure 1 A novel ERβ 5′-UTR

(**A**) 5′-RACE analyses of ERβ transcripts were performed; products representing a previously unidentified 5′-UTR containing sequence from or immediately upstream of exon 1 were amplified. Products from two breast cell lines are shown along with a diagram representing ERβ exon 1 (genomic locations with respect to the translational start are indicated). (**B**) Alignment of the 5′ end of the human ERβ gene with mRNAs containing either UTRa, UTRc or the novel UTR-E1. Coding regions (black boxes), transcriptional start sites (black arrows) and primers used for the qPCR analysis of each 5′-UTR (black arrows) are shown. The grey box indicates sequence included in a subset of transcripts with UTR-E1. (**C**) Relative expression of UTRa, UTRc and UTR-E1 in breast cell lines was examined by qPCR; all three cell lines examined expressed UTR-E1.

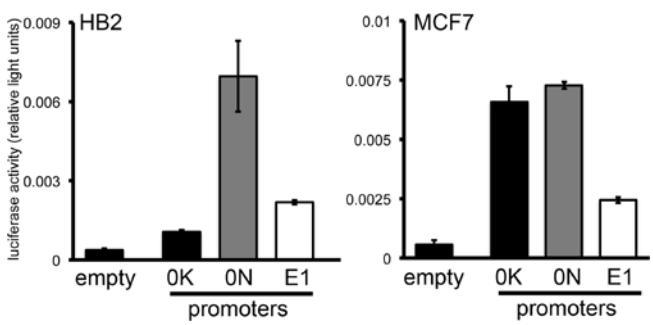

Figure 2 A third ERβ promoter shows activity in breast cell lines

Approx. 2 kb of genomic DNA directly upstream of exons 0K, 0N or E1 was cloned into luciferase reporter vectors. HB2 and MCF7 cell lines were transiently transfected with equal copy numbers of luciferase reporter lacking additional promoter sequences (empty) or containing promoter sequences as shown, and luciferase assays were performed after 24 h. A minimum of two independent experiments were performed, and within each experiment three technical replicates were included. Results are means ± S.D. of technical triplicates within a representative experiment.

by a third, previously uncharacterized, promoter that determines the expression of UTR-E1.

5′-UTRs differentially regulate efficiency of ERβ translation

We have shown previously that UTRa and UTRc have profound and differential influences on ERβ translation. We hypothesized that UTR-E1 may also influence translation, thus we extended our previous analyses to test this using our established GFP reporter assay [16,21]. UTRa, UTRc and UTR-E1 were cloned upstream of the GFP reading frame in expression vectors. For UTR-E1, we cloned the sequence encoded by the published extent of exon 1, representing a commonly identified 5′-RACE product. Cells were transiently transfected with equal numbers of copies of vectors to allow expression of GFP mRNAs either with non-regulatory 5′-UTRs (positive control; 'con'), or with UTRa, UTRc or UTR-E1. GFP protein expression was measured by flow cytometry, and GFP mRNA expression was measured by qPCR allowing determination of relative translational efficiencies for each GFP mRNA (Figure 3). As published previously [16], UTRa and UTRc inhibited translation, with UTRa being strikingly inhibitory in nature. UTR-E1 was also inhibitory in nature despite being short (only 90 nucleotides; as compared with UTRa, 289 nucleotides, and UTRc, 418 nucleotides) and having a very low predicted degree of secondary structure [as assessed by the theoretical change in free energy of folding, ΔG; UTR-E1's predicted ΔG is -14 kcal/mol (1 kcal = 4.184 kJ), whereas, for comparison, those

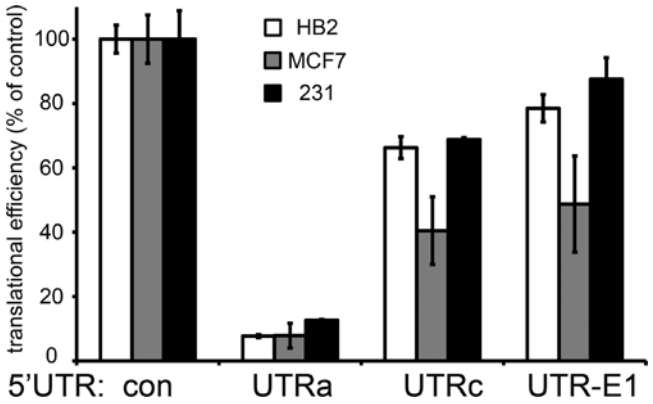

Figure 3 5′-UTRs regulate ERβ translational efficiency

Reporters were constructed to express mRNAs containing the GFP reading frame preceded by different 5′-UTRs: a control (con) sequence lacking regulatory motifs, UTRa, UTRc or UTR-E1. Cell lines were transiently transfected with equal copy numbers of either control or experimental constructs. GFP protein and mRNA were quantified by flow cytometry and real-time PCR respectively. Translational efficiency (protein synthesized per unit of mRNA) is presented relative to the GFP control that lacks a specialized 5′-UTR. A minimum of two independent experiments were performed and within each experiment three technical replicates were included. Results are means ± S.D. of technical triplicates within a representative experiment.

of UTRa and UTRc are −84 and −166 kcal/mol]. A consistent pattern of relative influences for each UTR was seen in all cell lines, with UTRa being more, and UTR-E1 less, inhibitory. We concluded that ERβ 5′-UTRs specified the efficiencies with which downstream ORFs are translated. We also noted that promoter E1 may be especially important in terms of defining ERβ function, since transcripts from this promoter, which contain UTR-E1, are the most efficiently translated ERβ mRNAs.

Cross-talk between 5′- and 3′-UTRs influences translation of individual ERβ isoforms

Transcripts for at least three functionally distinct ERβ isoforms are produced in breast cells [15]. These are derived from differential 3′ splicing of the final ERβ exons [4]. It is evident that this differential splicing must also confer different 3′-UTRs on transcripts for each isoform, although the exact 3′-UTR sequences are poorly defined, especially with respect to which potential polyadenylation sites are used. We were interested to examine whether ERβ 3′-UTRs might influence translation. First, we performed 3′-RACE analyses for each isoform in HB2 and MCF7 breast cells to identify 3′-UTR sequences (Figure 4A). For ERβ1, we were only able to amplify a product in MCF7 cells, probably because ERβ1 expression levels were low in HB2 cells. The 3′-UTR identified was of 242 nucleotides, representing a considerable 3′ extension of the published sequence (108 nucleotides; GenBank® accession NM_001437.2). For ERβ2, we identified three 3′-UTRs of similar lengths: an 85 nucleotide sequence in HB2 cells and 103 or 108 nucleotide sequences in MCF7 cells; each apparently terminating at polyadenylation sites slightly more proximal than the published 3′-UTR sequence of 120 nucleotides (GenBank® accession number AF051428). Cell-type-specific alternative 3′-UTRs were identified for ERβ5: sequences of 234 nucleotides in HB2 and of 79 nucleotides in MCF7 cells. These represented the use of either proximal or distal polyadenylation sites as compared with the published sequence of 177 nucleotides (GenBank® accession numbers DQ838583.1 and AF061055.1). We did not detect expression of an extended ERβ2 3′-UTR that is represented within GenBank® (accession number NM_001040276.1; 498 nucleotides). However, RACE reactions can be biased towards amplification of shorter sequences, therefore we designed primers to assess expression of this specific

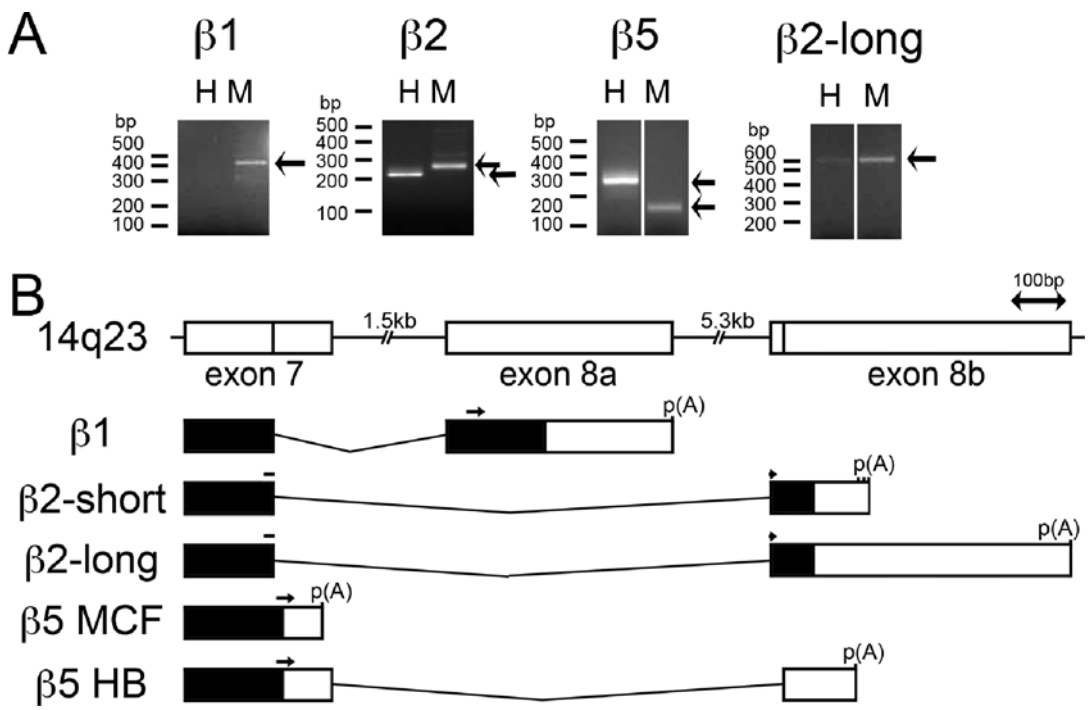

Figure 4 Differential splicing confers multiple and different 3′-UTRs on transcripts for each ERβ isoform

(**A**) 3′-RACE analyses for each ERβ isoform were performed on the breast cell lines HB2 (H) and MCF7 (M). Products were identified and sequenced. PCR analysis was also performed using primers specific for an ERβ2 3′-UTR that is represented within GenBank® (β2-long; right-hand panel). (**B**) Alignment of the 3′ end of the human ERβ gene with mRNAs for each isoform containing the 3′-UTRs (open boxes) identified. Coding regions (black boxes) and the location of polyadenylation site [p(A)] and primers used for amplification of products in (**A**) (black arrows) are shown.

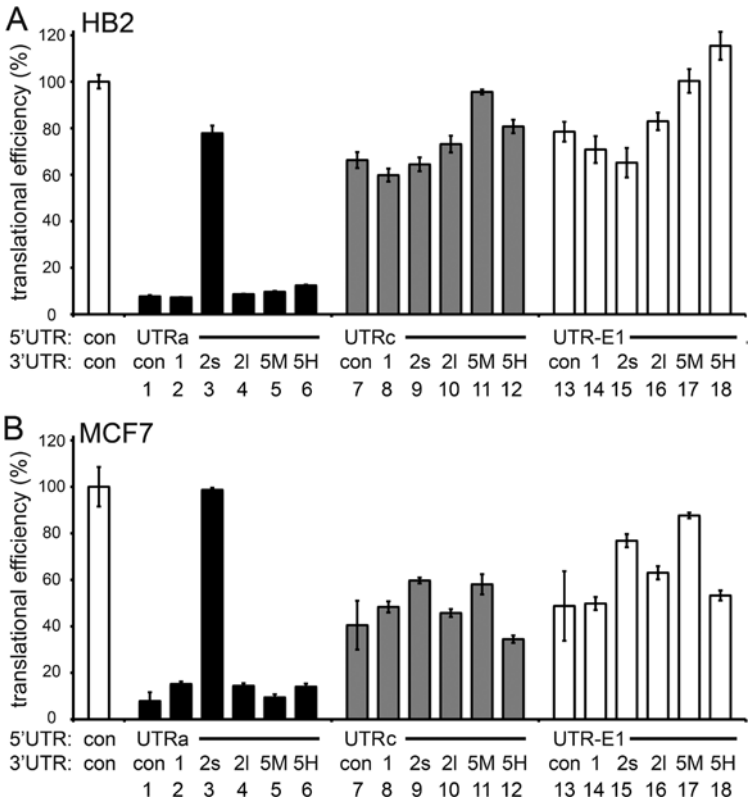

Figure 5 Isoform-specific 3′-UTRs define different translational efficiencies in conjunction with 5′-UTRs

3′-UTRs were cloned downstream of the GFP reading frame in each 5′-UTR GFP reporter construct. HB2 (**A**) and MCF7 (**B**) cells were transiently transfected with reporter constructs and translational efficiencies determined relative to the GFP control. A minimum of two independent experiments were performed and within each experiment three technical replicates were included. Results are means ± S.D. of technical triplicates within a representative experiment.

3′-UTR. The longer ERβ2 3′-UTR ('β2-long') was, in fact, expressed in both cell lines (Figure 4A; right-hand panel). These data are summarized in Figure 4(B) in the form of an alignment of the 3′ ends of mRNAs for each isoform with the 3′ end of the human ERβ gene.

We were interested to examine whether these isoform-specific 3′-UTRs define different translational efficiencies in conjunction with the 5′-UTRs, thereby allowing differential expression of the isoforms. We cloned 3′-UTRs downstream of the GFP reading frame in each 5′-UTR GFP reporter construct. For ERβ2, we focused on comparison of β2-long, as detected by specific PCR, with the sequences identified using RACE ('β2-short'; we examined a 120 nucleotide sequence, GenBank® accession number AF051428). For ERβ5, we examined both sequences identified by RACE ('β5 HB' and 'β5 MCF'). HB2 and MCF7 cells were transiently transfected with equal numbers of copies of vectors to allow expression of GFP mRNAs with non-regulatory UTRs (positive control; 'con'), or with reporters for each isoform-specific 3′-UTR in combination with each 5′-UTR. Translational efficiencies were determined as described above and are presented relative to positive controls (Figure 5). 3′-UTRs had potent and differential influences on translational efficiencies. The ERβ1 3′-UTR had little influence on the translational efficiencies specified by the 5′-UTRs in either cell line (compare lane 2 with lane 1, lane 8 with lane 7 and lane 14 with lane 13). In contrast, the two alternative ERβ2 3′-UTRs had markedly different effects. β2-long had little influence on the translational efficiencies specified by 5′-UTRs (compare lane 4 with lane 1, lane 10 with lane 7 and lane 16 with lane 13). However, β2-short induced dramatic and, in the case of MCF7 cells, total derepression of the translation inhibition specified by 5′-UTRa (compare lane 3 with lane 1; $P < 0.0001$ in both cell lines), but had relatively little influence when paired with UTRc or UTR-E1 (compare lane 9 with lane 7 and lane 15 with lane 13). ERβ5 3′-UTRs had smaller influences on translational efficiencies. β5 MCF induced derepression of the inhibitory influence of both UTRc and UTR-E1 (compare lane 11 with lane 7 and lane 13 with lane 8; $P < 0.02$ in all cases), whereas β5 HB had little influence except for inducing strong translation when paired with UTR-E1 (compare lane 18 with lane 13) in only HB2 cells (notably, these are the cells from which this 3′-UTR was cloned). We concluded that cross-talk between 5′- and 3′-UTRs had profound influences on the translational efficiency of transcripts for individual ERβ isoforms, with length of 3′-UTRs, as defined by differential use of polyadenylation sites, being a critical factor in determining the outcome.

E2 modifies ERβ transcription and translation

E2 influences the downstream effects of ERs by binding to and modifying their activity as transcription factors. It has also been reported that E2 has an impact on expression of ERβ itself at both transcriptional [22,23] and translational [24] levels by largely unknown mechanisms. We next investigated the influence of E2 on ERβ transcription and translation using our reporters for both of these regulatory stages. Cells were transfected with equal copy numbers of either luciferase reporters containing different ERβ promoters (as in Figure 2) or GFP reporters containing isoform-specific UTR pairings (as in Figure 5) for analysis of influences on transcription and translation respectively. Cells were then treated with vehicle or E2 for 24 h and were

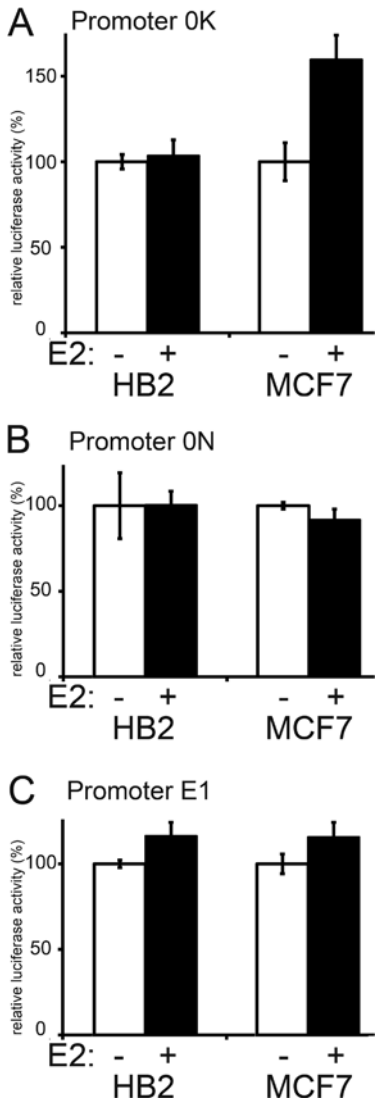

Figure 6 E2 modifies ERβ transcription

HB2 and MCF7 cells were transfected with equal copy numbers of luciferase reporters containing different ERβ promoters (as in Figure 2). Cells were treated with vehicle (white bars) or 10 nM E2 (black bars) for 24 h, and luciferase assays were performed. E2 caused an increase in the activity of promoter 0K in MCF7 cells, but not in HB2 cells (**A**), whereas it had no significant effect on promoters 0N or E1 (**B** and **C**). A minimum of two independent experiments were performed and within each experiment three technical replicates were included. Results are means $\pm$ S.D. of technical triplicates within a representative experiment.

analysed for luciferase activity or translational efficiency of GFP transcripts. In terms of transcriptional activity, E2 caused a 1.6-fold activation of the 0K promoter in MCF7 cells ($P = 0.001$), but not in HB2 cells (Figure 6A), while having no significant effect on promoters 0N or E1 (Figures 6B and 6C). Effects of E2 on translational efficiencies specified by the various UTR pairings were more complex. Translation of transcripts containing the ERβ1 3′-UTR were increased by E2 when combined with UTRc or UTR-E1 (Figure 7A; $P = 0.008$ and 0.03 respectively). ERβ2 3′-UTRs (both short and long) specified an E2-dependent decrease in translational efficiency when paired with 5′-UTRa (Figure 7B; both $P = 0.003$), but an increase when paired with UTR-E1 ($P = 0.01$ and 0.049 respectively). ERβ5 3′-UTRs also specified differential responses to E2, determining both decreases (β5 MCF paired with UTR-E1; $P = 0.005$) and increases in

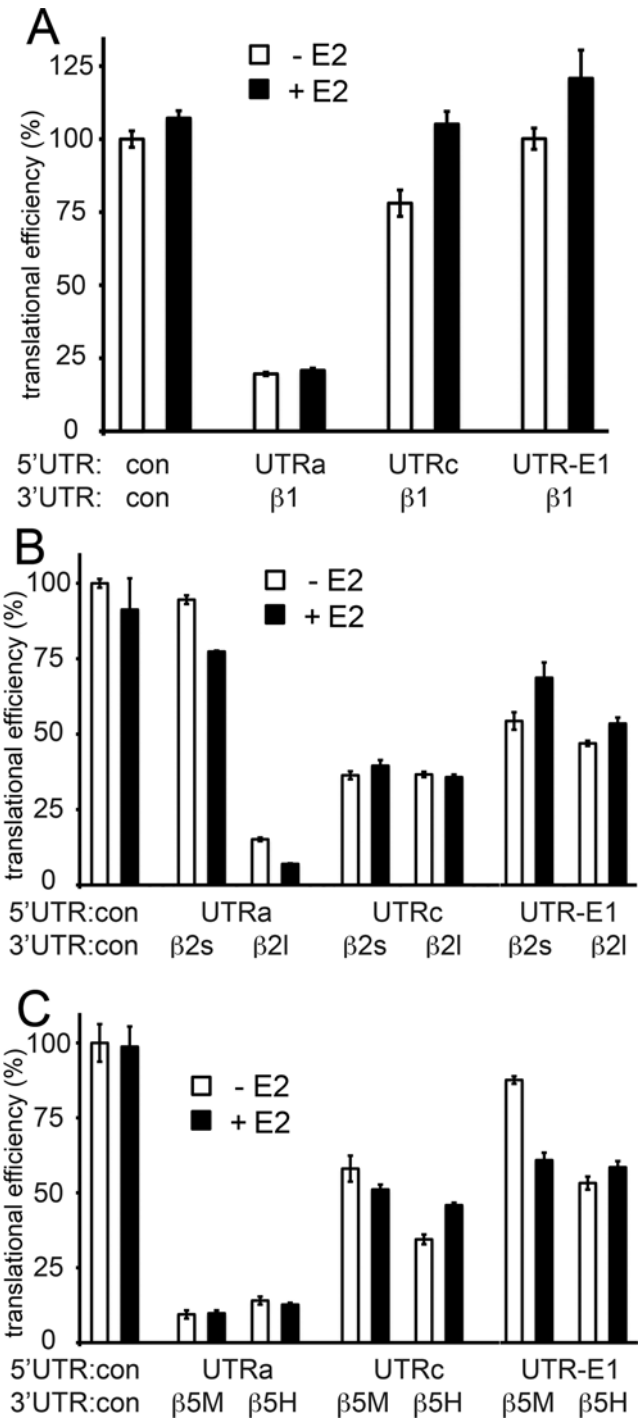

Figure 7 E2 modifies ERβ translation

MCF7 cells were transfected with equal copy numbers of GFP reporters containing UTR pairings (as in Figure 5). Cells were treated with vehicle (white bars) or 10 nM E2 (black bars) for 24 h, and translational efficiency of GFP transcripts was determined. A minimum of two independent experiments were performed, and within each experiment three technical replicates were included. Results are means $\pm$ S.D. of technical triplicates within a representative experiment.

translation (β5 HB paired with UTRc; $P = 0.002$). We concluded that E2 has only relatively weak influences on ERβ transcription and translation. However, as these influences act differentially on the promoters and on the isoform-specific UTR pairings, these weak influences cumulatively have potential to modify the balance of ERβ isoforms.

DISCUSSION

It is well established that the ERβ gene has two promoters allowing the expression of full-length transcripts, promoters 0K and 0N [20]. mRNAs transcribed from each promoter have different 5′-UTRs, termed UTRa and UTRc [16] (Figure 1B). However, full-length ERβ transcripts containing neither of these UTRs have been reported [25], and the presence of an additional promoter, giving rise to further alternative 5′-UTRs, has long been suspected [26]. We have identified an additional 5′-UTR, UTR-E1, and the corresponding novel promoter region, promoter E1 (Figures 1 and 2). A number of previous studies have attempted to assess the relative transcriptional strengths and thereby the importance of promoters 0K and 0N [27,28]. However, our previous data, demonstrating that ERβ 5′-UTRs determine differential and cell-type-specific translational inhibition [16], cast doubt on these assessments. Reporter assays including exonic sequences would combine translational differences with the expected measure of transcription. We have therefore investigated promoter activities of only sequences immediately upstream of the exons thereby avoiding translational regulatory motifs (although risking failure to include important transcription-factor-binding sites within the exons), and have analysed translational regulation separately. We found promoter 0N to be the most active in both cell lines tested, although promoters E1 and 0K are also active (Figure 2). Methylation of CpG islands within promoter 0N has been reported in cancers, including those of the breast [29], ovary [30] and prostate [28], and this is thought to be responsible for down-regulation of some forms of ERβ during carcinogenesis [9,10,31]. In this context, it is interesting to note the presence of a CpG island within promoter E1 (from −2180 to −1878 with respect to the translational start site); our preliminary data suggest that these sequences are also commonly methylated in breast cancers [32]. It is also interesting that the relative activities of the promoters as determined by luciferase assay (Figure 2) do not correlate with the expression of the 5′-UTRs derived from the endogenous promoters (Figure 1C). This may relate to the inhibitory methylation of endogenous promoters, or to the influence of transcription factors binding outside the sequence included in the reporter constructs.

We demonstrated previously that ERβ expression is regulated at the level of translation, with UTRa and UTRc having potent and differential influences [16]. When these analyses were extended to include UTR-E1, we found that UTR-E1 allowed the most efficient translation of the ERβ 5′-UTRs (Figure 3). Therefore transcripts from promoter E1, which contain UTR-E1, are likely to contribute disproportionally to ERβ protein levels. Moreover, a key finding of the present study is that translational regulation acts differentially on transcripts for the ERβ isoforms 1, 2 and 5. These transcripts are derived from differential 3′ splicing [4], giving them isoform-specific 3′-UTRs. We identified 3′-UTR sequences for each isoform (Figure 4) and demonstrated that, when combined with ERβ 5′-UTRs, these specified a wide range of translational efficiencies (Figure 5). Most striking was the ability of different ERβ2 3′-UTRs to define up to a 10-fold difference in translational efficiency when paired with UTRa. 5′-UTRa inhibits translation on account of inefficient translational scanning induced by secondary structure and initiation at uORFs (upstream ORFs) [16]. The shorter ERβ2 3′-UTR (β2-short) overcame this inhibitory influence, whereas the longer form (β2-long), which included the entire sequence of β2-short, had little effect. Regulatory elements in both 5′- and 3′-UTRs have important roles in determining translational efficiencies, facilitated by transcript circularization induced by interaction of poly(A)-binding protein binding at the 3′ end of transcripts with eIF4G (eukaryotic initiation factor 4G) at 5′ ends [33,34]. β2-short apparently interacted with 5′-UTRa, resulting in a loss of inhibitory structures and/or uORF translation, whereas the same sequences were unable to interact in this way in the context of β2-long. Therefore differential use of polyadenylation sites, defining 3′-UTR length, exerts translational control. It is interesting that faster-proliferating cells and cancer cells preferentially use proximal polyadenylation sites [35,36]; in the case of ERβ2, this would lead to increased translation that may explain how ERβ2 is up-regulated in breast cancer cells [10,14] despite promoter methylation and down-regulation of ERβ transcripts [29,37]. Shortening of 3′-UTRs has been associated with increased protein expression for a number of cancer-related genes [36]. For these genes, this resulted from increased mRNA stability and/or loss of microRNA-mediated translational repression when compared with their longer 3′-UTRs. For ERβ2, this is clearly not the case, since β2-short dominantly derepressed the influence of 5′-UTRa, as opposed to merely lacking repressive 3′ elements present in β2-long. ERβ5 3′-UTRs also tended to specify relatively efficient translation, but, in this case, only when modifying the influences of 5′-UTRc or UTR-E1. In MCF7 cells, this was only apparent for the ERβ5 3′-UTR that was actually cloned from these cells. As for ERβ2, the data do not support a simple model of shorter 3′-UTRs having fewer repressive elements, since both lengths of ERβ5 3′-UTR acted to derepress the influences of inhibitory 5′-UTRs.

Finally, we investigated influences of the ER ligand E2 on transcriptional activity of ERβ promoters and translation specified by ERβ UTRs. We found E2 exerted relatively mild, although statistically significant, influences at multiple levels, with potential to change the balance of ERβ isoform expression. Unfortunately, we were unable to confirm whether this balance is altered, since quantitative detection of endogenous ERβ protein isoforms in cell lines is unreliable with the antibodies currently available. First, we found E2 induced transcription in MCF7 cells only with the 0K promoter (Figure 6), a promoter we have shown previously to exhibit a relative preference for producing ERβ1 and ERβ5 transcripts in this cell type [16]. Secondly, we found E2 induced a wide range of changes in translational efficiencies of transcripts with ERβ UTR pairs (Figure 7); the overall effect in any cell type would be defined by the relative proportions of each UTR pairing within the total pool of transcripts for that isoform. E2 is known to modulate levels of microRNAs [38,39], thus these constitute potential mediators of its influence on ERβ translational efficiencies. Indeed, we have found that *miR-92* can regulate expression of ERβ1 acting at its 3′-UTR [40]. However, we find that different UTR pairs respond differently, therefore we infer that accessibility of individual microRNA-binding sites in the context of interactions between 5′- and 3′-UTRs would influence whether particular transcripts respond to changes in levels of any particular microRNA.

In conclusion, we have revealed novel mechanisms controlling ERβ expression that help explain the reported lack of concordance between ERβ mRNA and protein levels, and the differential expression of ERβ1, ERβ2 and ERβ5, and give new insights into the regulation of ER function. To the best of our knowledge, this is the first time cross-talk between multiple 5′- and 3′-UTRs has been implicated in the differential regulation of translation of different protein isoforms from one gene, demonstrating how the ERβ gene provides a model for study of complex gene regulatory pathways.

AUTHOR CONTRIBUTION

Thomas Hughes and Laura Smith devised the study and wrote the paper. Laura Smith carried out the vast majority of experimental work. Louise Coleman, Sampoorna Satheesha and

Spencer Shaw cloned and analysed the luciferase reporters. Michele Cummings analysed promoter sequences *in silico*. Valerie Speirs advised on the design of the experiments. All authors edited the paper before submission.

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MOLECULAR CANCER

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Response to mTOR inhibition: activity of eIF4E predicts sensitivity in cell lines and acquired changes in eIF4E regulation in breast cancer

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Abstract

Background: Inhibitors of the kinase mTOR, such as rapamycin and everolimus, have been used as cancer therapeutics with limited success since some tumours are resistant. Efforts to establish predictive markers to allow selection of patients with tumours likely to respond have centred on determining phosphorylation states of mTOR or its targets 4E-BP1 and S6K in cancer cells. In an alternative approach we estimated eIF4E activity, a key effector of mTOR function, and tested the hypothesis that eIF4E activity predicts sensitivity to mTOR inhibition in cell lines and in breast tumours.

Results: We found a greater than three fold difference in sensitivity of representative colon, lung and breast cell lines to rapamycin. Using an assay to quantify influences of eIF4E on the translational efficiency specified by structured 5'UTRs, we showed that this estimate of eIF4E activity was a significant predictor of rapamycin sensitivity, with higher eIF4E activities indicative of enhanced sensitivity. Surprisingly, non-transformed cell lines were not less sensitive to rapamycin and did not have lower eIF4E activities than cancer lines, suggesting the mTOR/4E-BP1/eIF4E axis is deregulated in these non-transformed cells. In the context of clinical breast cancers, we estimated eIF4E activity by analysing expression of eIF4E and its functional regulators within tumour cells and combining these scores to reflect inhibitory and activating influences on eIF4E. Estimates of eIF4E activity in cancer biopsies taken at diagnosis did not predict sensitivity to 11-14 days of pre-operative everolimus treatment, as assessed by change in tumour cell proliferation from diagnosis to surgical excision. However, higher pre-treatment eIF4E activity was significantly associated with dramatic post-treatment changes in expression of eIF4E and 4E-binding proteins, suggesting that eIF4E is further deregulated in these tumours in response to mTOR inhibition.

Conclusions: Estimates of eIF4E activity predict sensitivity to mTOR inhibition in cell lines but breast tumours with high estimated eIF4E activity gain changes in eIF4E regulation in order to enhance resistance.

Background

Rapamycin is an immunosuppressant drug prescribed for prophylaxis of organ rejection following renal transplant [1]. Recently it, and derivatives such as everolimus, have been tested as cancer therapeutics with some success [2-5]. The drugs inhibit the serine/threonine-specific protein kinase mTOR (mammalian Target Of Rapamycin) by forming a complex with another protein, FKBP12 (FK 506-binding protein of 12 kDa), that then associates with mTOR. This association allosterically inhibits mTOR's ability to assemble the functionally active complex mTORC1 (mTOR complex 1) [6,7]. In addition, at high doses the drugs can bind directly to mTOR inhibiting its function [8]. mTORC1 activity is up-regulated in many cancers as a result of loss of function of tumour suppressor genes such as p53 or LKB1, up-regulation of AKT, or mitogenic signalling [9-11]. Pathways downstream of mTORC1 that contribute to carcinogenesis have also been defined. The main mTORC1 targets are the eIF4E-binding proteins (4E-BP1, 2 and 3) and the S6 protein

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kinases (S6K1 and 2) [12,13]. Hypophosphorylated 4E-BPs bind to and inhibit the translation factor eIF4E, while these interactions are inhibited by mTORC1-dependent 4E-BP phosphorylation, releasing active eIF4E [14]. S6K activity is stimulated by phosphorylation by mTORC1. The result of increased activity of both eIF4E and S6K is changes in translation. Increased eIF4E activity enhances cap-dependent translation of mRNAs with a high degree of secondary structure within their 5' untranslated regions (UTRs) [15,16], a subset of transcripts greatly enriched for cancer-related messages [17]. In addition, nuclear export of some cancer-related transcripts is stimulated by highly active eIF4E [18,19]. Increased S6K activity leads to up-regulation of overall translational capacity, as a result of increased ribosome biogenesis, and may also contribute to enhanced translation of transcripts with structured 5'UTRs via up-regulation of the activity of the translation factor eIF4A [20]. Therefore, increased mTORC1 activity in cancer enhances expression of key oncogenes and increases cellular growth potential. Reversing these effects, and thereby reducing cell growth or inducing apoptosis, is thought to be the basis of the therapeutic action of mTOR inhibitors in cancer.

However, mTOR inhibitors have proved less successful in cancer clinical trials than might be hoped from the importance of the molecular pathways involved [2]. This relates partly to some toxicity in non-target tissues [21,22], but also to intrinsic or acquired resistance in many individual cancers. Consequently, there is a need for predictive biomarkers to allow selection of patients with cancers most likely to respond to such agents. A number of potential biomarkers have been discussed in the literature, focusing on expression levels or phosphorylation states of mTOR itself [23], or the immediate targets of mTORC1, 4E-BP1 [24,25] and S6K1 [26,27]. Here, we take a different approach and estimate the *activity* of eIF4E, one of the key effectors of mTORC1 function, and investigate whether this reflects response to mTOR inhibition in both tissue culture and in clinical breast cancers.

Methods

Cell culture, transfection, proliferation assays

Cell lines were obtained from American Tissue Culture Collection or European Collection of Animal Cell Cultures and were maintained at 37°C in humidified air/5% CO_2. Bi-monthly mycoplasma checks (MycoAlert assay; Lonza, Basel, Switzerland) were consistently negative. Cell-specific culture/transfection conditions are described in Additional file 1, Table S1. Plasmids pTH-GFPa (control GFP reporter) [28], GFP+60 (structured GFP reporter) [29] and pcDNA3HA-eIF4E [30] have been described previously. For proliferation assays, cells were plated into 96-well, flat-bottomed plates at 5×10^3-2×10^4 cells/well (depending on cell line growth characteristic to ensure continued log phase growth; this was assessed by examination of growth of DMSO-treated cells over 48 h). Five replicate wells were treated with DMSO (control) or InSolution™ Rapamycin (Calbiochem, Darmstadt, Germany) for 24 (data not shown) or 48 h. Metabolically active cells were quantified by assessing conversion of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma-Aldrich, Dorset, UK) to formazan. Formazan was dissolved in propan-1-ol and quantified as absorbance at 570 nm (Opsys, Dynex, Sussex, UK).

Western blotting

Proteins were extracted in RIPA (50 mM Tris HCl pH 7.4, 150 mM NaCl, NP40 1%, Complete inhibitors [Roche, Basel, Switzerland]) and were quantified in triplicate with the RCDC protein assay (BioRad, Hercules, USA). 20 μg of protein was loaded into wells of 12% NuPAGE bis-tris gels running in MOPS NuPAGE buffer (Invitrogen, Paisley, UK). Proteins were transferred to PDVF membrane (Millipore, Billerica, USA) in NuPAGE transfer buffer (Invitrogen, Paisley, UK). Membranes were blocked and incubated with antibodies in 5% dried milk in TBS-T (Tris-buffered saline-0.1% Tween 20) and were washed in TBS-T. Primary antibodies: rabbit monoclonal anti-phosphoThr37/46 4E-BP1, 1:500, and rabbit polyclonal anti-4E-BP1, 1:500 (#2855 and #9452, Cell Signalling Technology, Beverly, USA); mouse monoclonal anti-eIF4E, 1:500 (sc9976, Santa Cruz, USA). We have previously validated specificities of these antibodies, including the phospho-specificity of the anti-phospho clone [31], although we cannot exclude that the anti-phospho-4E-BP1 may cross-react with phospho-4E-BP2 or 3. Secondary antibodies: anti-mouse/rabbit HRP conjugates, 1:1000 (DAKO, Glostrup, Denmark). Proteins were detected using Supersignal West Femto (Thermo Fisher, Waltham, USA) and Chemidoc XRS (BioRad, Hercules, USA), and analysed using ImageJ 1.42q (NIH, http://rsb.info.nih.gov/ij).

Translational efficiency assay

We have previously reported the protocol in considerable detail [32,33]. In brief, relative GFP protein and mRNA levels were used to calculate amounts of GFP protein produced per unit mRNA. RNA was purified using RNeasy (Qiagen, Crawley, UK) and contaminating DNA was removed with TURBO DNase I (Applied Biosystems, Warrington, UK). cDNA was synthesized from oligo-dT primed RNA using Superscript II (Invitrogen, Paisley, UK). Real-time PCR reactions were performed in triplicate using SYBR Green PCR Master Mix on an ABI7900HT machine (Applied Biosystems, Warrington,

UK). GFP mRNA levels were normalized to those of the reference gene RPLP0 [34] and relative expression calculated using the ΔΔCt method [35]. For analysis of GFP protein expression, cells were suspended in media containing 1% serum and fluorescence quantified (mean fluorescent intensity of 10^4 events after exclusion of debris/dead cells on forward activated light scatter/side scatter) at 525 nm using an LSRII machine (BD Biosciences, Oxford, UK).

Ethical issues, patient material, immunohistochemistry

Ethical permissions were obtained from Northern and Yorkshire MREC (4/MRE03/89) and Leeds East REC (05/Q1206/136). Postmenopausal female patients with operable early breast cancer (T1-3, N0-1, M0) proceeding to primary surgery were recruited, written informed consent was taken, and patients were treated as previously described in detail [36]. In brief, core biopsies were taken at time of presentation with a palpable breast lump and were processed for diagnostic assessments. Patients were given 11-14 days of everolimus 5 mg once daily immediately before tumour resection. Excision specimens were processed by the pathology laboratory for diagnostic tests. Clinical/pathological details of patients are listed elsewhere [36]. Matched biopsy and excisional tumour blocks from 22 patients were used. Immunohistochemistry was performed for Ki67 (mouse monoclonal clone MIB-1; Dako, Glostrup, Denmark), 4E-BP2 (rabbit polyclonal #2845, Cell Signalling Technology, Beverly, USA), eIF4E, 4E-BP1 and phosphoThr37/46 4E-BP1 (as for Westerns) exactly as described and validated previously [31,36] on single sections from each case for each antigen. Ki67 was quantified using a previously validated protocol for scoring percentages of stained cells as proportions of total cancer cells [37], and these data have been published [36]. Other markers were scored by two independent individuals (VJC, CS) taking into account average intensity and percentage of positively stained tumour cells. Intensity scores (0 no staining, 1 weak, 2 moderate and 3 strong) were added to percentages scores (1 <5%, 2 6-25%, 3 26-75% and 4 >75%) giving totals of 0 or 2-7. Consensus scores were determined for sections with different initial scores; scoring was overseen by a consultant breast histopathologist (AMH).

Statistics

Analyses were performed using Student's T Test, Spearman's rho correlation, or linear regression in Excel v12 (Microsoft, Redmond, USA), SPSS v15 (SPSS, Chicago, USA) and MATLAB (MathWorks, Natick, USA). Tests were two sided; $p < 0.05$ was considered to indicate significance.

Results

Cell lines show a range of sensitivities to rapamycin

Rapamycin and its derivatives induce a broad range of responses when used as cancer therapeutics with growth of some cancers reduced while others are resistant. We were interested to examine this variation, therefore we treated a panel of cell lines with rapamycin and determined drug sensitivities. The panel was representative of the three most common cancers in the UK: colorectal (SW480 and Caco2, moderately-differentiated and heterogeneous colon cancer lines respectively); lung (U2020, small cell, and H1299 and A549, non small cell cancer lines); and breast (MCF7 and MDA-MB-231, luminal and basal breast cancer lines respectively). In addition, two immortal breast epithelial lines of non-cancer origin (HB2 and MCF10A) were examined in order to allow study of potential differential sensitivity between cancer and non-cancer cells. We treated cells with doses of rapamycin and determined proliferation/survival relative to control treated cells using MTT assays after 48 h (Figure 1A). Sensitivities to the highest dose are shown in Figure 1B. As expected a range of sensitivities were seen, with a three fold difference between the most sensitive (MCF7) and most resistant (MDA-MB-231). Cells of non-cancer origin (HB2 and MCF10A) were found to have sensitivities between these extremes.

The phosphorylation state of 4E-BP1 does not predict rapamycin sensitivity

Next, we aimed to identify molecular markers that correlated with these sensitivities, therefore that might represent predictive biomarkers for mTOR inhibitors. Potential biomarkers have previously been proposed; of particular interest was the phosphorylation status of 4E-BP1 [24] since the 4E-BP1/eIF4E axis has been shown to be critical for mTOR-mediated transformation [38]. 4E-BP1 is directly phosphorylated by mTORC1 [12], potentially leading to increased eIF4E activity and enhanced translation of cancer-related transcripts [15-17]. Thus, levels of phosphorylated 4E-BP1 may reflect contributions of mTORC1-signalling to cancer-associated translational deregulation, and consequently the sensitivity of such deregulated cells to mTOR inhibition. We performed Western blot analysis of levels of mTORC1-dependent 4E-BP1 phosphorylation (Thr37/Thr46) in the same cell lines as before (Figure 2A). At least three different phosphorylated 4E-BP1 (phospho-4E-BP1) species were seen, representing various combinations of the seven potential phosphorylation events [39-41]. We found no correlation between phospho-4E-BP1 and rapamycin sensitivity (compare Figures 2A and 1B). However, levels of phospho-4E-BP1 reflect not

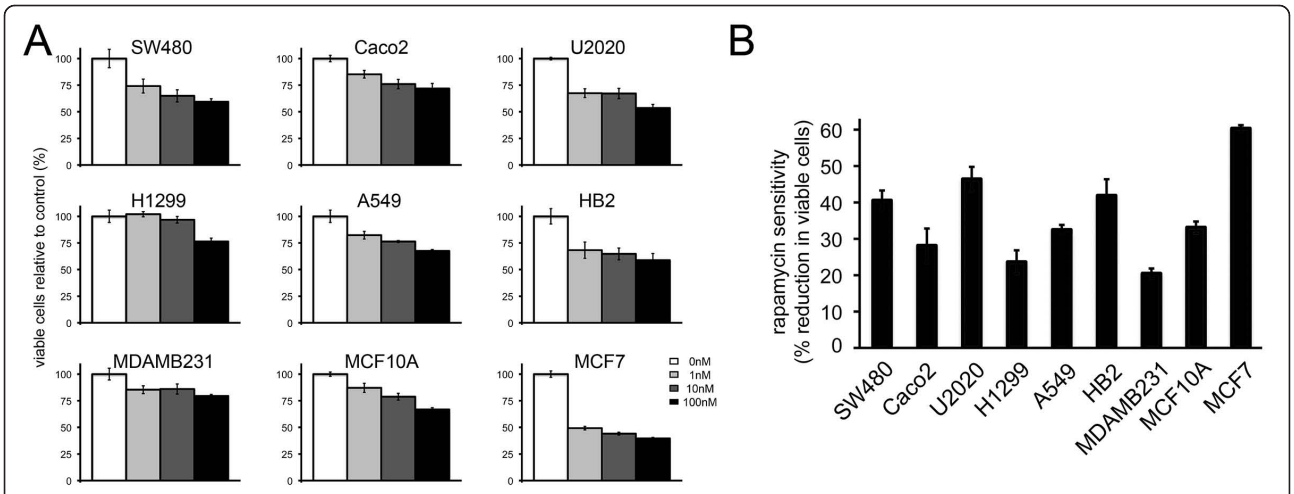

Figure 1 Sensitivities of cell lines to rapamycin vary by up to three fold. A) Cells were treated with either control, or different doses of rapamycin and growth/proliferation was monitored using MTT assays. MTT readings after 48 hours are shown relative to control. B) Relative sensitivities to rapamycin are shown; these are the % reductions in growth/proliferation caused by 100 nM rapamycin. Data points represent means (+/- standard deviations) from five independent wells of cells within a representative experiment. Data from independent repeat experiments are presented in Additional file 2, Figure S1 to demonstrate the reproducibility of these measurements in each cell line.

only mTORC1 activity but also levels of overall 4E-BP1, therefore we also analysed total 4E-BP1 expression (Figure 2B) and determined the ratios of phospho- to total 4E-BP1 (Figure 2C) as a measure of mTORC1's influence on 4E-BP1 function, as previously reported [25]. We found no correlation between this measure and rapamycin sensitivity. Finally, 4E-BP1's influence on cellular behaviour is determined by the amount of eIF4E remaining unbound by 4E-BP1, consequently, variation in eIF4E expression would also have a critical role. We analysed eIF4E expression and found greater than three fold variation in eIF4E expression (Figure 2D). We concluded that levels of phospho-4E-BP1 do not correlate with functional influences of mTORC1 on cap-dependent translation, partly as substantial variations in total 4E-BP1 and eIF4E expression mask this direct relationship. A measure of eIF4E activity would, however, take into account the variation in expression of all these components and might provide a better predictive marker.

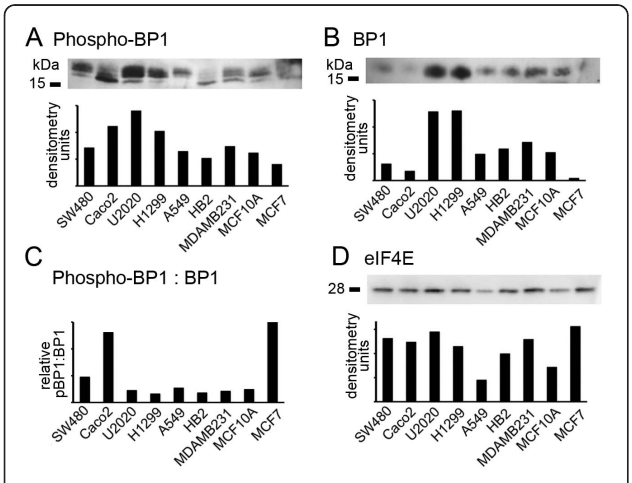

Figure 2 Levels of 4E-BP1 phosphorylation do not predict rapamycin sensitivity. Expression of phospho-4E-BP1 (A), total 4E-BP1 (B) and eIF4E (D) was examined in the cell lines shown by Western blot. Relative ratios of phospho-4E-BP1 to total 4E-BP1 are shown in C. Equal masses of total protein were loaded in each lane as determined by triplicate measurements with colourimetric protein mass assays, and expression was quantified by densitometry.

Assessment of translational efficiencies defined by a structured 5'UTR; estimation of eIF4E activity

Our hypothesis was that assessment of the *activity* of a key mTORC1 regulated pathway gives direct insights into the contribution of deregulated mTORC1 to cellular behaviour and therefore, potentially, into likely sensitivity to mTOR-inhibitors. A critical effect of up-regulated mTORC1 is to up-regulate eIF4E activity thereby enhancing translational efficiencies of transcripts with structured 5'UTRs [15]; therefore, we designed an assay to measure these translational efficiencies. We have previously shown that a 5'UTR from human axin2 transcripts contains a sixty nucleotide sequence that is predicted to form a stable stem-loop structure [29]. This sequences meets the criteria associated with UTRs that determine differential translational efficiencies in response to changes in eIF4E activity [16], while lacking other translation regulatory motifs (e.g. upstream AUG codons or binding sites for trans-acting proteins). In addition, we previously demonstrated that this sequence

determined cell type specific translational efficiencies [29]. We now wished to examine whether the translational efficiency defined by this sequence would respond to changes in eIF4E activity, and could therefore be representative of mTORC1's influence on cap-dependent translation of structured transcripts. The sequence was cloned upstream of the GFP reading frame in an expression vector. MCF7 cells were transiently transfected with an equal copy number of vectors to allow expression of GFP mRNAs with either a control non-regulatory 5'UTR or this sequence as a 5'UTR, along with either empty expression plasmids or plasmids allowing eIF4E over-expression. GFP protein expression was measured by flow-cytometry and GFP mRNA expression was measured by qPCR allowing determination of relative translational efficiencies for each GFP message as previously described [32,33] (Figure 3A). Western blot analyses were used to confirm expression of exogenous eIF4E and GFP (Figure 3B). The translational efficiency of the control reporter was not significantly altered by eIF4E over-expression (compare lanes 1 and 2), demonstrating that eIF4E over-expression did not cause a general enhancement of translation. As previously reported [29], the structured 5'UTR conferred repression of translation (compare lanes 1 and 3; $p = 0.002$). Critically, this repression was overcome by exogenous eIF4E (compare lanes 3 and 4; $p = 0.002$), resulting in translation with the same efficiency as messages lacking inhibitory 5'UTRs. We concluded that this reporter did indeed respond to changes in eIF4E activity and thus can be used to provide an estimate of eIF4E-dependent translation from structured 5'UTRs.

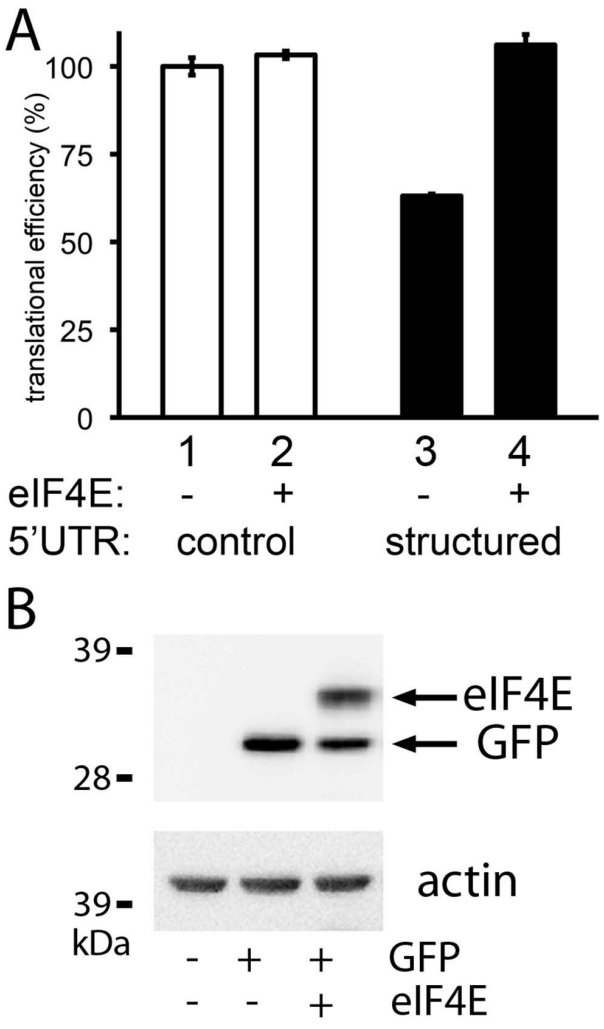

Figure 3 Translational efficiency specified by a structured 5'UTR reporter responds to changes in eIF4E activity.
A) Reporters were constructed to express mRNAs containing the GFP reading frame preceded by a control 5'UTR lacking regulatory motifs (control) or a sequence predicted to form a stable stem-loop structure (structured). MCF7 cells were transiently transfected with equal copy numbers of either control or structured reporters along with either empty expression vector (-) or vector to allow over-expression of eIF4E (+). GFP protein and mRNA were quantified by flow-cytometry and real-time PCR respectively. Translational efficiency (protein synthesised per unit mRNA) is presented relative to the control. Data points represent means (+/-standard deviations) of values from three separate wells of cells within a representative experiment. B) Expression of exogenous proteins was confirmed within cells transfected with control GFP reporter and vector to allow over-expression of eIF4E as shown by Western blot analysis for the HA-epitope tag; exogenous GFP and eIF4E both include this epitope. Note that the small reduction in GFP protein associated with eIF4E-transfection does not result from a change in translational efficiency (see Panel A).

Estimates of eIF4E activity predict rapamycin sensitivity in tissue culture cells

Relative translational efficiencies specified by this eIF4E-responsive 5'UTR were determined in the panel of cell lines. Cells were transiently transfected with vectors to allow expression of GFP mRNAs with control or the structured 5'UTR as before, and translational efficiencies were determined (Figure 4A). A range of translational efficiencies was seen, with A549 cells determining the lowest, and HB2 cells the highest (Figure 4B). Surprisingly, the two cells lines of non-cancer origin (HB2 and MCF10A) were found to determine relatively efficient translation from the reporter 5'UTR. Importantly, translational efficiencies specified by the eIF4E-responsive 5'UTR correlated with rapamycin sensitivity (Figure 5). Initially, we analysed this relationship using Spearman's rank correlation coefficient; we found a strong and significant positive association ($r = 0.72$; $p = 0.037$).

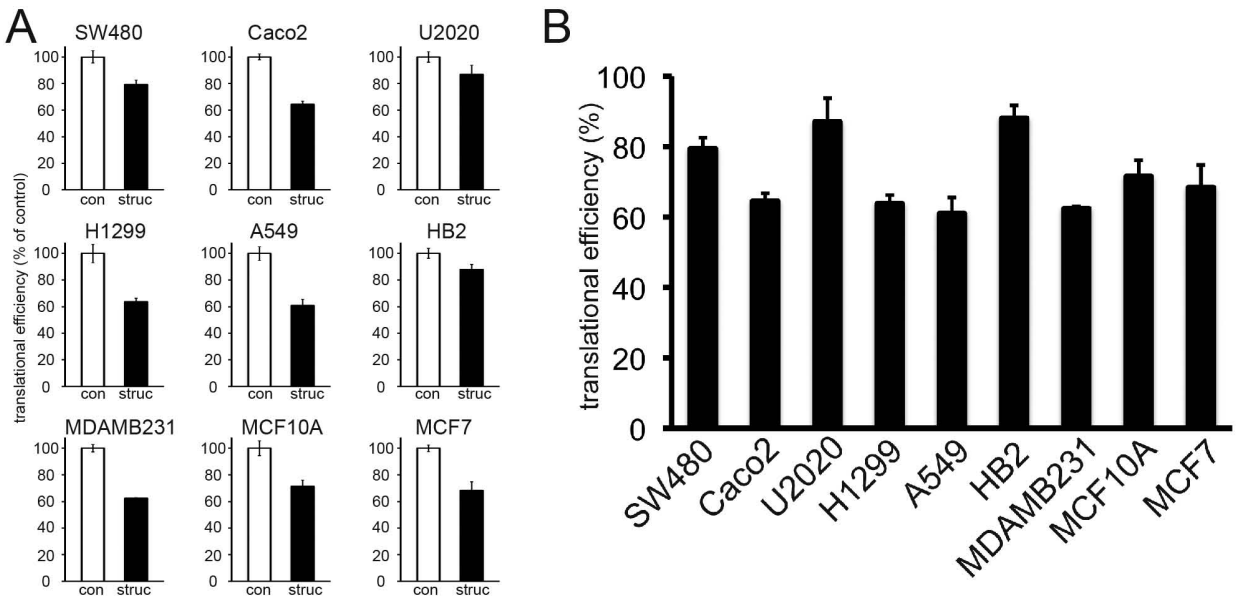

Figure 4 eIF4E activities vary in different cell lines. A) Cells were transiently transfected with equal copy numbers of plasmids to allow expression of transcripts with the GFP reading frame preceded by either a control 5'UTR lacking regulatory motifs (con) or the eIF4E-responsive structured 5'UTR (struc). GFP protein and mRNA were quantified by flow-cytometry and real-time PCR respectively. Translational efficiency (protein synthesised per unit mRNA) is presented relative to the control. Data points represent means (+/-standard deviations) of values from three separate wells of cells from two independent experiments (a total of six values). B) Translational efficiencies of transcripts with eIF4E-responsive 5'UTRs in the 9 cells lines.

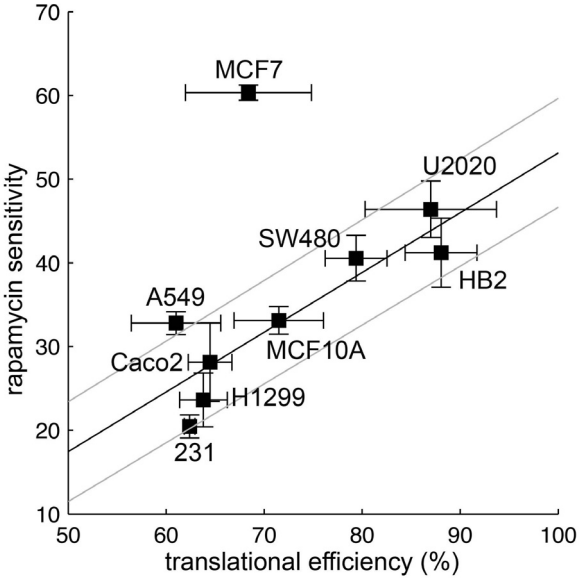

Figure 5 Experimentally determined eIF4E activities correlate with sensitivities to rapamycin. Data from Figures 4B and 1B were plotted for each cell line as labelled. Linear regression was performed to determine the relationship in the cell lines excluding the outlier, MCF7 cells; the linear model is shown as a line (black) with 95% confidence intervals (grey lines) (p = 0.0037).

However, the correlation was particularly evident in 8/9 cell lines; if MCF7 cells, which were more sensitive to rapamycin than predicted, were excluded the strength and significance of the relationship was increased (r = 0.83; p = 0.015). Similarly in linear regression, a highly significant relationship was seen when MCF7 cells were excluded from the analysis (Figure 5; p = 0.0037).

Estimated eIF4E activity in breast tumours does not predict reduced tumour proliferation after preoperative treatment with the rapamycin derivative everolimus

Next, we wished to examine whether eIF4E activities within tumour cells predict clinical responses to mTOR inhibition in cancer patients, and whether changes in eIF4E activities after treatment reflect these responses. Everolimus is a rapamycin-derivative with improved oral bioavailability that is currently undergoing trials as a cancer therapeutic. We recently performed a clinical trial examining effects of everolimus on tumour cell proliferation when given preoperatively as a single agent to breast cancer patients [36]. By examining Ki67 staining in matched pre-treatment biopsy and post-treatment excisional samples, we found that 5 mg everolimus daily for up to 14 days was significantly associated with reduced tumour cell proliferation. Unfortunately, it is not possible to estimate eIF4E activities directly in tumour samples such as these using the reporters

described above. As an alternative we estimated eIF4E activity from expression and phosphorylation states of multiple regulators of the translational initiation pathway (Figure 6; Table 1). We have previously described the development and prognostic value of this estimate in breast tumours [31]. Expression levels of eIF4E, 4E-BP1, 4E-BP2 and Thr37/Thr46 phosphorylated 4E-BP1 (phospho-4E-BP1) within tumour cells were determined semi-quantitatively in matched pre- and post-treatment tumour samples from 22 patients using immunohistochemistry. Activity of eIF4E was estimated by combining these scores to reflect the inhibitory influence of 4E-BPs on eIF4E, and the activating influence of 4E-BP1 phosphorylation on eIF4E, using a formula previously derived from regression modelling of individual contributions of each component to prognosis in a large cohort of breast cancer patients: X-B1/4+PB1/2-B2/4, where X, B1, PB1 and B2 represent scores for eIF4E, 4E-BP1 phospho-4E-BP1 and 4E-BP2 respectively [31]. We previously showed that this measure gave improved insights into the prognostic influence of eIF4E by reflecting eIF4E activity more accurately than examining eIF4E expression levels alone.

17/22 tumours showed reduced Ki67 scores after treatment (mean reduction 48%) indicating apparent responses to everolimus (Table 1). Disappointingly, estimates of pre-treatment eIF4E activity did not predict the occurrence or extent of these responses. Similarly, pre-treatment phospho-4E-BP1 levels had no predictive value. Estimated eIF4E activity was, however, reduced in post-treatment samples (mean change in score -1.7; range -8 to +1.25; $p < 0.001$) but surprisingly this was not attributable to reduced phospho-4E-BP1. Phospho-4E-BP1 expression was reduced after treatment (mean -2.3; range -6 to +1; $p < 0.001$), suggesting a reduction in mTOR-dependent phosphorylation of 4E-BP1, but this reduction in phospho-4E-BP1 was not significantly correlated with the reduction in estimated eIF4E activity, and changes in levels of the other components had strong influences on estimated eIF4E activity. For example, 4E-BP1 expression changed considerably (mean -0.3; range -5 to +5; $p = 0.01$), meaning that 8 individual decreases in phospho-4E-BP1 could be explained at least partially by reductions in total 4E-BP1, as opposed to reduced phosphorylation. This explanation is supported by observations that the phospho-4E-BP1 species examined here (Thr37/46) can be relatively resistant to mTOR inhibition [39]. 4E-BP2 expression also frequently changed (mean +2.2; range -2 to +7; $p < 0.001$), while some individuals showed dramatic changes in expression of eIF4E (mean -0.1; range -6 to +4). Interestingly changes in eIF4E and 4E-BP1 were positively associated ($r = 0.60$, $p = 0.003$), often resulting in relatively small changes in estimated eIF4E activity, despite substantial fluctuations in expression of the individual proteins. Critically, neither reduced estimated eIF4E activity or reduced phospho-4E-BP1 correlated with reduced Ki67.

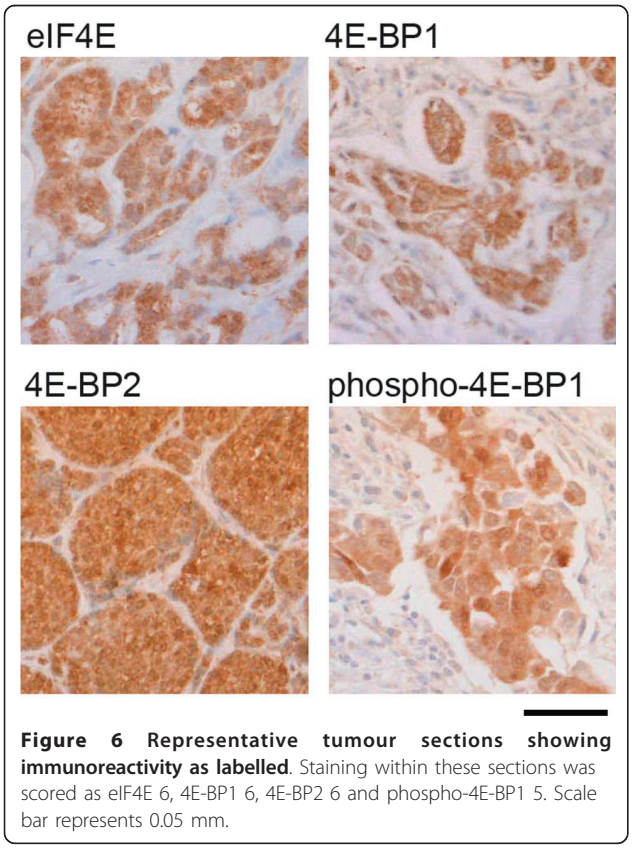

Figure 6 Representative tumour sections showing immunoreactivity as labelled. Staining within these sections was scored as eIF4E 6, 4E-BP1 6, 4E-BP2 6 and phospho-4E-BP1 5. Scale bar represents 0.05 mm.

High estimated eIF4E activities in breast tumours are associated with everolimus-induced changes in eIF4E regulation

A striking observation was that post-treatment levels of eIF4E and the 4E-BPs frequently varied considerably from pre-treatment levels. Our hypothesis was that these changes represent development of acquired resistance to inhibition of eIF4E activity, by induction of changes in eIF4E regulation within tumour cells or by clonal selection of cells with different eIF4E-related expression profiles. Importantly, this proposed acquired resistance is not necessarily reflected in higher proliferation rates. Using this hypothesis, one would predict that tumours with high pre-treatment estimated eIF4E activities would be most subject to drug-induced expression changes or to clonal selection pressures, and would show the greatest adaptive changes in eIF4E regulation. In fact, high estimated eIF4E activity pre-treatment did positively correlate with the combined magnitude of changes in expression

Table 1 Expression scores for eIF4E (4E), 4E-BP1 (BP1), 4E-BP2 (BP2), phospho-4E-BP1 (pBP1) and Ki67, and estimates of eIF4E activity in matched pre- and post-treatment samples from patients treated with 11-14 days of everolimus

	pre-treatment						post-treatment					
Patient	4E	BP1	BP2	pBP1	activity	Ki67	4E	BP1	BP2	pBP1	activity	Ki67
1	6	5	5	6	6.5	15.5	0	0	6	0	-1.5	23.1
2	3	5	5	5	3	6.0	0	3	5	0	-2	0.9
3	3	3	5	4	3	7.6	5	4	5	3	4.25	2.7
4	5	6	6	6	5	22.1	6	7	7	6	5.5	16.7
5	4	6	4	6	4.5	44.6	4	4	4	3	3.5	29.7
6	4	0	6	6	5.5	34.2	5	5	5	4	4.5	23.0
7	3	6	5	5	1.75	4.2	4	4	4	0	2	2.7
8	4	3	4	2	3.25	1.6	3	0	4	3	3.5	4.4
9	5	7	7	6	4.5	15.3	5	6	7	5	4.25	6.8
10	5	7	6	5	4.25	22.6	5	3	6	2	3.75	6.5
11	5	0	6	2	4.5	18.4	5	0	4	0	4	7.4
12	5	3	6	6	5.75	3.6	6	6	7	3	4.25	47.2
13	4	4	0	5	5.5	10.1	6	5	5	2	4.5	3.6
14	6	4	0	6	8	17.4	4	4	6	2	2.5	10.1
15	5	0	0	6	8	10.2	4	0	7	5	4.75	17.8
16	5	4	0	4	6	10.6	6	4	6	2	4.5	13.0
17	4	4	2	6	5.5	16.2	6	3	5	4	6	8.4
18	0	0	0	4	2	6.2	4	2	7	0	1.75	5.2
19	4	4	0	5	5.5	18.2	7	7	5	3	5.5	9.0
20	6	4	0	7	8.5	18.0	5	6	7	5	4.25	3.8
21	5	4	6	6	5.5	20.0	0	0	5	5	1.25	13.4
22	5	4	2	4	5.5	6.8	4	4	7	3	2.75	5.3

of the four markers ($r = 0.46$, $p = 0.03$) and, in particular, with increases in 4E-BP2 expression ($r = 0.55$, $p < 0.01$). We concluded that high estimated eIF4E activity may, in fact, predict tumour response to everolimus; however this response is not the expected reduction in proliferation, but is development of changes in eIF4E regulation, presumably to promote everolimus resistance.

Discussion

The mTOR pathway, which promotes cell proliferation, presents an attractive target for cancer therapy since it is deregulated in a wide range of cancer types and a large proportion of cases of each type [2]. However, resistance of some cancers to mTOR-directed therapeutics has limited the success of mTOR inhibitors. We have examined this variation in response, initially, in cell lines. As expected, and in accordance with other published work [42-44], we found a range of sensitivities to rapamycin (Figure 1). Surprisingly, we found that cancer cell lines were not more sensitive than cells of non-cancer origin, despite the well established preferential sensitivity of cancer cells over non-cancer cells in animal models and, to an extent, in humans [3-5]. This observation most likely demonstrates that up-regulation of mTORC1, and consequently sensitivity to its inhibition, is actually associated with growth as opposed to malignancy, and therefore that highly-proliferative, immortal, non-cancer cell lines are un-representative of 'normal', relatively slowly growing, epithelial cells with respect to mTORC1 signalling. The efficacy of rapamycin as an immunosuppressant drug [1] and the side-effects seen in cancer therapies [21,22] support the view that proliferating cells are targeted.

Identification of predictive biomarkers for mTOR-targeted therapies such as rapamycin or everolimus has become a research focus [2]. Levels of phosphorylated mTOR, S6K1 or 4E-BP1 have been seen as logical markers as these phosphorylation events induce mTOR activity or are directly catalysed by mTORC1, and therefore levels may reflect the extent of mTORC1 deregulation. However, in principle, it is obvious that levels of these species may not correlate directly with their influences on down-stream signalling and consequent changes in cellular behaviour, since these influences would also be defined by expression/activity of the other regulatory molecules of the pathways. Despite this, some predictive value has been demonstrated for each marker [23,24,27]. We found levels of phosphorylated 4E-BP1, and the proportions of phospho-4E-BP1 within the total

pool of 4E-BP1 to be unrelated to rapamycin sensitivity in tissue culture, in accordance with previous work in a cell line panel also containing MCF7 and MDA-MB-231 cells [42]. Moreover, we showed that this, in fact, was the expected result in the context of variation in 4E-BP1 and eIF4E expression (Figure 2). As an alternative predictive marker, we developed assays to estimate one of the key functional end-points of mTORC1 signalling, eIF4E *activity*. We found this estimate to be significantly associated with rapamycin sensitivity in cell culture. It was notable, however, that estimated eIF4E activity was the most significant predictor of rapamycin sensitivity for 8 of the cell lines (Figure 5), while MCF7 cells were twice as sensitive as predicted by this relationship. MCF7 cells over-express S6K1, on account of amplification of its gene [45]; one explanation for enhanced sensitivity in MCF7 cells may be that with constitutively high S6K1 activity, the cells are dependent upon mTOR-induced S6K functions such as more general translational effects [46]. In support of this, S6K1 overexpression has previously been associated with increased rapamycin sensitivity [42].

Importantly, we also examined whether estimates of pre-treatment eIF4E activity in clinical breast tumours predicted response to the mTOR inhibitor everolimus. Disappointingly and in contrast to our *in vitro* work, we found estimated eIF4E activity did not predict response to mTOR inhibition as assessed by change in tumour cell proliferation. However, we did find that pre-treatment eIF4E activity in tumours was significantly associated with substantial changes in the expression of eIF4E and its regulators post-treatment. We interpret this to suggest that cancers with high eIF4E activity may indeed have been sensitive to everolimus, as suggested by our *in vitro* data, but that the cells remaining after two weeks of drug treatment reflect selection to acquire drug resistance by changing the pathways regulating eIF4E function. Data show that this proposed resistance is not necessarily associated with lower estimated eIF4E activity or higher proliferative rates. This hypothesis highlights a difference between short-term (two day) sensitivity assays *in vitro* and longer term (two week) drug treatments in patients; in the latter case it is inevitably more difficult to assess the early response of tumour cells to treatment and there is considerable scope for acquired changes to take place. Finally, it is interesting to note that our data do not support the use of phospho-4E-BP1 as either a predictive or pharmacodynamic marker for mTOR inhibitors as some have attempted [2,21] since it is clear that changes in phospho-4E-BP1 relate not only to inhibition of 4E-BP1 phosphorylation, but also to dramatic changes in overall 4E-BP1 expression.

Additional material

Additional file 1: Table S1. Cell culture and transfection conditions.

Additional file 2: Figure S1. Sensitivities of cell lines to rapamycin are reproducible. Relative sensitivities to 100 nM rapamycin are shown; these are the % reductions in growth/proliferation caused by the drug as compared to control treated cells. Data from Figure 1B are reproduced (filled bars) alongside independent repeat analyses (open bars). Data points represent means (+/- standard deviations) from five independent wells of cells.

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Authors' contributions
SS, VJC, LJC and NI performed the *in vitro* experiments. BM and JNM performed statistical analyses. CABS, VJC and AMH performed semi-quantitative scoring of tissue sections. VSS, EJM, JMSB, JMD established the everolimus clinical trial, and obtained/analysed clinical samples. TAH devised and directed the study. All authors contributed to writing the manuscript and have approved the final version.

Competing interests
JMD possesses an unrestricted educational grant and honoraria for seminar presentations from Novartis Pharma. The other authors declare that they have no conflicts of interest.

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